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Research Paper

The synergistic effect between hydrogen peroxide and nitrite, two long-lived molecular species from cold atmospheric plasma, triggers tumor cells to induce their own cell death

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ABSTRACT

Nitrite and H_2O_2 are long-lived species in cold atmospheric plasma and plasma-activated medium. It is known that their synergistic interaction is required for selective apoptosis induction in tumor cells that are treated with plasma-activated medium. This study shows that the interaction between nitrite and H_2O_2 leads to the formation of peroxynitrite, followed by singlet oxygen generation through the interaction between peroxynitrite and residual H_2O_2. This primary singlet oxygen causes local inactivation of few catalase molecules on the surface of tumor cells. As a consequence, H_2O_2 and peroxynitrite that are constantly produced by tumor cells and are usually decomposed by their protective membrane-associated catalase, are surviving at the site of locally inactivated catalase. This leads to the generation of secondary singlet oxygen through the interaction between tumor cell-derived H_2O_2 and peroxynitrite. This selfsustained process leads to autoamplification of secondary singlet oxygen generation and catalase inactivation. Inactivation of catalase allows the influx of H_2O_2 through aquaporins, leading to intracellular glutathione depletion and sensitization of the cells for apoptosis induction through lipid peroxidation. It also allows to establish intercellular apoptosis-inducing HOCl signaling, driven by active NOX1 and finalized by lipid peroxidation through hydroxyl radicals that activates the mitochondrial pathway of apoptosis. This experimentally established model is based on a triggering function of CAP and PAM-derived H_2O_2/nitrite that causes selective cell death in tumor cells based on their own ROS and RNS. This model explains the selectivity of CAP and PAM action towards tumor cells and is in contradiction to previous models that implicated that ROS/RNS from CAP or PAM were sufficient to directly cause cell death of tumor cells.

1. Introduction

1.1. Cold atmospheric plasma (CAP) and plasma-activated medium (PAM)

Cold atmospheric plasma (CAP) and its liquid phase contain electrons, photons, as well as superoxide anions, hydroperoxyl radicals, H_2O_2, hydroxyl radicals, atomic oxygen, singlet oxygen, ozone, nitric oxide, nitrogen dioxide, peroxynitrite, nitrite, nitrate, dichloride radicals and hypochloride anions[1–4],

Promising antitumor effects of CAP and PAM *in vitro* and *in vivo* have been studied in a very broad variety of tumor systems [reviewed in Refs. [5–18]]. In most studies, CAP and PAM were found to selectively cause cell death in malignant cells [reviewed in reference 1]. First encouraging results of clinical application of CAP for tumor therapy have been recently reported [19–21].

It has been recognized that among the many species contained in CAP or its liquid phase, particularly H_2O_2 and nitrite have a good chance to reach target cells that are covered by a layer of medium *in vitro,* or to cross substantial barriers of biological material during tumor treatment *in vivo* [22–26]. This is obviously not the case for highly reactive, and therefore short-ranging species from the liquid phase of CAP, such as peroxynitrite, ozone, hydroxyl radicals and singlet oxygen.

The long-lived and far-ranging molecular species H_2O_2 and nitrite are also the major biologically relevant constituents of plasma-activated medium (PAM) and plasma-activated buffer [27–32]. Girard et al. [28] and Kurake et al. [29] already recognized that the specific antitumor cell effect of PAM required the synergistic interaction between nitrite and H_2O_2. They also suggested that the generation of peroxynitrite through the reaction between nitrite and H_2O_2, following the reaction described by Lukes et al. [33], might play a central role for the observed biological effects. Their conclusions are in line with the suggestions by Jablonowski and von Woedtke [31].

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As both CAP and PAM cause apoptosis induction in tumor cells *in vitro* and *in vivo* [27–29,34–41], the combination of nitrite and H_2O_2 seems to be the lowest common denominator that is sufficient for selective apoptosis induction in tumor cells *in vitro* and *in vivo*. However, the mode of action towards tumor cells of these two relatively simple compounds had remained enigmatic so far.

1.2. Redox-related elements on the membranes of nonmalignant and malignant cells

The change from nonmalignant cells to transformed cells (early stage of oncogenesis) and further on to tumor cells (late stage of oncogenesis) is associated with relevant changes of the redox elements on the surface of these cells [42–50]. Malignant cells are distinguished from nonmalignant cells by sustained expression of membrane-associated NADPH oxidase (NOX1) [[51–54], reviewed in Refs. [48–50]]. NOX1-derived superoxide anions and their dismutation product H_2O_2 are required by transformed cells for autocrine stimulation of proliferation [51–54, reviewed in 48–50], Cells from late stage of oncogenesis, i. e. bona fide tumor cells, express catalase and SOD on their membranes, in addition to NOX1 [42,43,48–50,55–59]. Membrane-associated catalase protects the cells towards two NOX1-driven apoptosis-inducing signaling processes, i. e. the HOCl and the NO/peroxynitrite signaling pathway, which selectively eliminate malignant cells [42,44,45,50,60–64]. Catalase interferes with both pathways, as it efficiently decomposes H_2O_2, and thus prevents HOCl synthesis, and in addition oxidizes NO and decomposes peroxynitrite [42,48–50,64–67]. Membrane-associated SOD is required to prevent inhibition of catalase by abundant NOX1-derived superoxide anions [49,59]. Despite high local concentration of catalase on their membrane, tumor cells have lower concentrations of intracellular catalase [summarized in 43].Tumor cells are also characterized by a high concentration of aquaporins in their membrane [68].

This manuscript shows that the synergistic interaction between nitrite and H_2O_2 leads to the generation of low concentrations of primary singlet oxygen, which causes local inactivation of membrane-associated catalase. As a result, massive secondary singlet oxygen generation by the tumor cells, driven by sustained action of their NOX1 and NOS leads to inactivation of protective catalase and reactivation of intercellular apoptosis-inducing signaling. Therefore, ROS/RNS interactions in CAP and PAM are a specific trigger for tumor cells to contribute efficiently to their own cell death, driven by the ROS/RNS related chemical biology.

2. Materials and methods

2.1. Materials

The NOX1 inhibitor 4-(2-Aminoethyl)benzenesulfonyl fluoride (AEBSF), the aquaporin inhibitor $AgNO_3$, the catalase inhibitor 3-aminotriazole (3-AT), the inhibitor of glutathione synthesis buthionine sulfoximine (BSO), catalase from bovine liver, the hydroxyl radical scavenger dimethylthiourea, the fast decaying NO donor dieethylamine NONOate (DEA NONOate), glucose oxidase (GOX), the singlet oxygen scavenger histidine, hydrogen peroxyide, the hydroxyl radical scavenger mannitol, the NOS inhibitor N-omega-nitro-L-arginine methylester hydrochloride (L-NAME), the proton pump inhibitor omeprazole, potassium nitrite, the HOCl scavenger taurine, Mn-SOD from E. coli, were obtained from Sigma-Aldrich (Schnelldorf, Germany).

The peroxidase inhibitor 4-Aminobenzoyl hydrazide (ABH) was obtained from Acros Organics (Geel, Belgium). Inhibitors for caspase-3 (Z-DEVD-FMK), caspase-8 (Z-IETD-FMK) and caspase-9 (Z-LEHD-FMK) were obtained from R&D Systems (Wiesbaden-Nordenstadt, Germany).

Peroxynitrite and the peroxynitrite decomposition catalyst 5-, 10-, 15-, 20-Tetrakis(4-sulfonatophenyl)porphyrinato iron(III) chloride (FeTPPS), were obtained from Calbiochem (Merck Biosciences GmbH, Schwalbach/Ts, Germany).

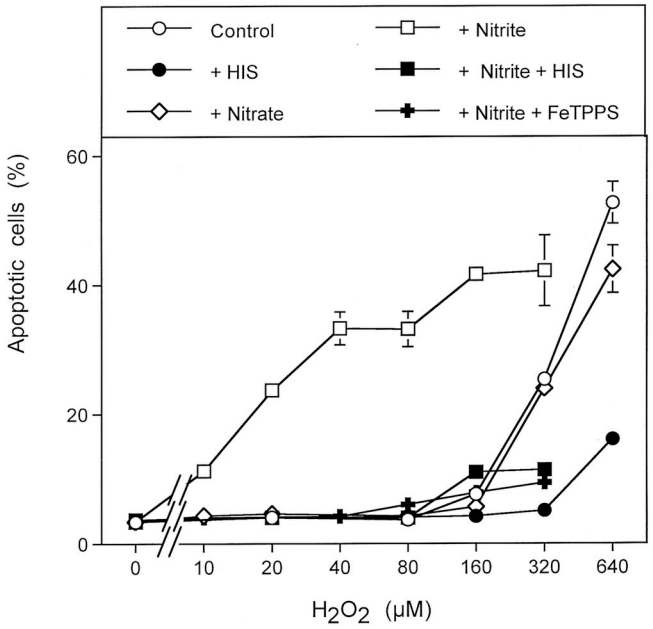


Fig. 1. The effect of H_2O_2 on tumor cells. Human gastric carcinoma cells MKN-45 were treated with increasing concentrations of H_2O_2 without further additions (control). Where indicated, assays received in addition either 2 mM of the singlet oxygen scavenger histidine, 1 mM of nitrite or nitrate, a combination of nitrite and histidine, or a combination of nitrite and 25 μM FeTPPS. All additions had been applied before addition of H_2O_2. The percentages of apoptotic cells were determined after 2.5 h.
The results show that concentrations of 320 μM H_2O_2 and more caused apoptosis induction that was dependent on singlet oxygen generation, in line with a previous report [47]. Lower concentrations synergized with nitrite, but not with nitrate. The synergistic effect between H_2O_2 and nitrite seemed to depend on singlet oxygen and peroxynitrite.
Statistical analysis: Apoptosis induction by H_2O_2, the synergistic effect between H_2O_2 and nitrite, as well as inhibition of apoptosis induction by histidine and FeTPPS were highly significant ($p < 0.001$). Nitrate caused no significant effect.

The catalase mimetic EUK-134 [chloro([2,2'-[1,2-ethanediylbis [(nitrilo-κN)methylidyne]]bis[6-methoxyphenolato-κO]]]-manganese was a product of Cayman (Ann Arbor, Michigan, U.S.A.) and was obtained from Biomol (Hamburg, Germany).

All small interfering RNAs (siRNAs) used in this study were obtained from Qiagen (Hilden, Germany) and are described in detail under Methods.

Detailed information on inhibitors has been previously published [55,56,61,69,75]. The site of action of inhibitors and scavengers has been presented in detail in the supplementary material of references [69,75].

2.2. Cells and media for cell culture

The human gastric adenocarcinoma cell line MKN-45 (ACC 409) (established from the poorly differentiated adenocarcinoma of the stomach (medullary type) of a 62 year-old woman), was purchased from DSMZ, Braunschweig, Germany. MKN-45 were cultured in RPMI 1640 medium, containing 10% fetal bovine serum (FBS).

Fetal bovine serum (Biochrom, Berlin, Germany) was heated for 30 min at 56 °C prior to use. Medium was supplemented with penicillin (40 U/ml), streptomycin (50 μg/ml), neomycin (10 μg/ml), moronal (10 U/ml) and glutamine (280 μg/ml). Care was taken to avoid cell densities below 300 000/ml and above 10^6/ml.

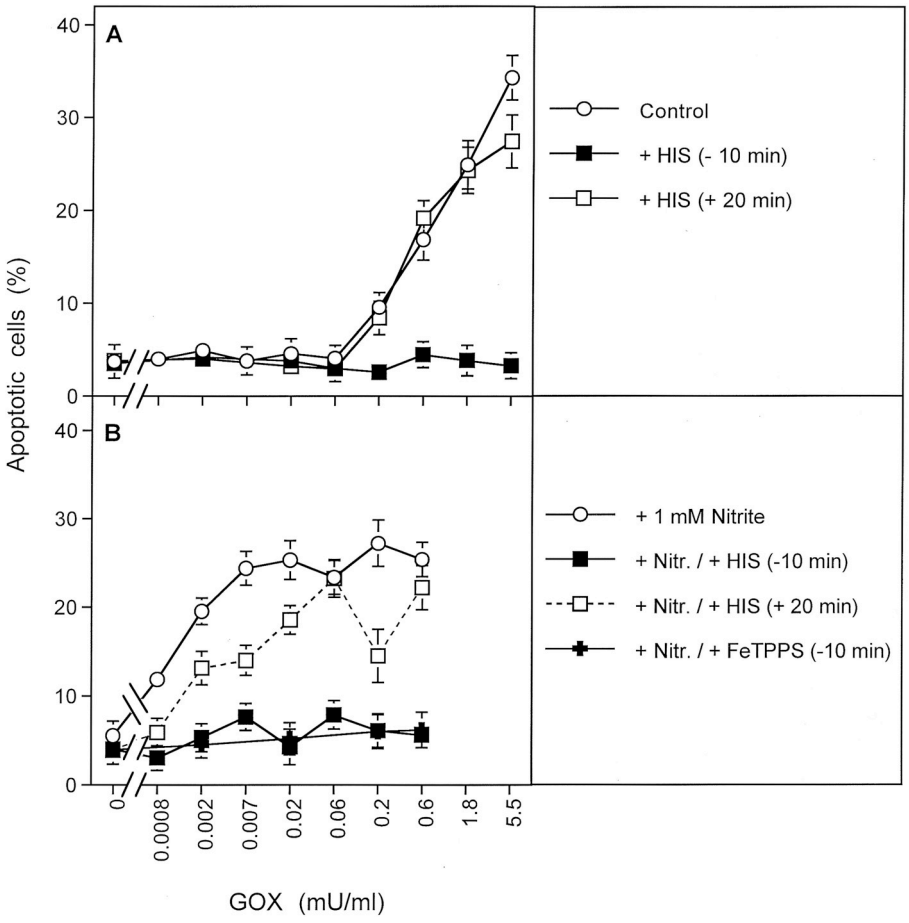


Fig. 2. The effect H_2O_2-generating glucose oxidase (GOX) on tumor cells. MKN-45 cells received the indicated concentrations of GOX, in the absence or presence of 1 mM nitrite. In addition, as indicated, histidine (2mM) or FeTPPS (25 μM) were added either 10 min before or 20 min after addition of GOX. The percentages of apoptotic cells were determined after 2.5 hrs.

The results show that GOX, as expected, mimicked the effect shown for H_2O_2 in the previous figure. They also show that the singlet oxygen-mediated step at very high concentrations of GOX and the singlet oxygen-mediated step at lower concentrations of GOX combined with nitrite were completed within short time.

Statistical analysis: Apoptosis induction mediated by GOX, the synergistic effect between GOX and nitrite, as well as inhibition of apoptosis induction by histidine or FeTPPS applied 10 min before GOX were highly significant ($p < 0.001$). The differences of the inhibitory effects by histidine dependent on the time of their application were highly significant ($p < 0.001$).

2.3. Methods

2.3.1. Autocrine apoptosis induction by intercellular ROS signaling

Cells in complete medium were seeded in 96-well tissue culture clusters at a density of 12 500 cells/100 μl. Reactivation of intercellular apoptosis-inducing ROS signaling required inactivation of membrane-associated catalase of tumor cells. Assays received the indicated concentrations of nitrite, GOX, H_2O_2 or a a combination of both, as indicated in the figures. In inhibitor studies, inhibitors were either applied 10 min before GOX/nitrite or at the indicated times after addition of GOX/nitrite. In all experiments, assays were performed in duplicate. After the indicated time of incubation at 37 °C and 5% CO_2 that allowed intercellular ROS-mediated apoptosis induction, the percentage of apoptotic cells was determined by inverted phase contrast microscopy based on classical criteria for apoptosis as described below.

2.4. Challenge with exogenous peroxynitrite for quantitation of membrane-associated catalase activity of tumor cells

Treatment with exogenous peroxynitrite allows to quantitatively monitor the activity of membrane-associated catalase as this enzyme decomposes exogenous peroxynitrite, whereas intracellular catalase cannot reach exogenous peroxynitrite before the compound attacks the cell membrane [43]. After the indicated pretreatments at a density of 125 000 cells/ml, the cells were washed several times through centrifugation and resuspension in fresh medium and then were seeded at a density of 12 500 cells/100 μl. The cells 100 μl received 100 μM AEBSF to prevent autocrine apoptosis induction and negative interference of cell-derived H_2O_2 with peroxynitrite. Peroxynitrite was diluted in ice-cold PBS immediately after controlled thawing and was rapidly applied to the cells. This approach allows to focus on apoptosis induction by exogenous peroxynitrite which is an indication for the inactivation of membrane-associated catalase. Apoptosis criteria were used as defined below.

2.5. Determination of the percentage of apoptotic cells

After the indicated time of incubation at 37 °C and 5% CO_2, the percentage of apoptotic cells was determined by inverted phase contrast microscopy based on the classical criteria for apoptosis, i.e., nuclear condensation/fragmentation or membrane blebbing [42,49,70,71,75]. The characteristic morphological features of intact and apoptotic cells, as determined by inverted phase contrast microscopy have been published [42,72–75]. At least 200 neighbouring cells from randomly selected areas were scored for the percentage of apoptotic cells at each point of measurement. Control assays ensured that the morphological features 'nuclear condensation/fragmentation' as determined by inverse phase contrast microscopy were correlated to intense staining with bisbenzimide and to DNA strand breaks, detectable by the TUNEL reaction [61,72–74]. A recent systematic comparison of methods for the quantitation of apoptotic cells has shown that there is a perfect coherence between the pattern of cells with condensed/fragmented nuclei (stained with bisbenzimide) and TUNEL-positive cells in assays with substantial apoptosis induction, whereas there was no significant nuclear condensation/fragmentation in control assays [74,75]. Further controls ensured that ROS-mediated apoptosis induction was mediated by the mitochondrial pathway of apoptosis, involving caspase-9 and caspase-3 [63,74].

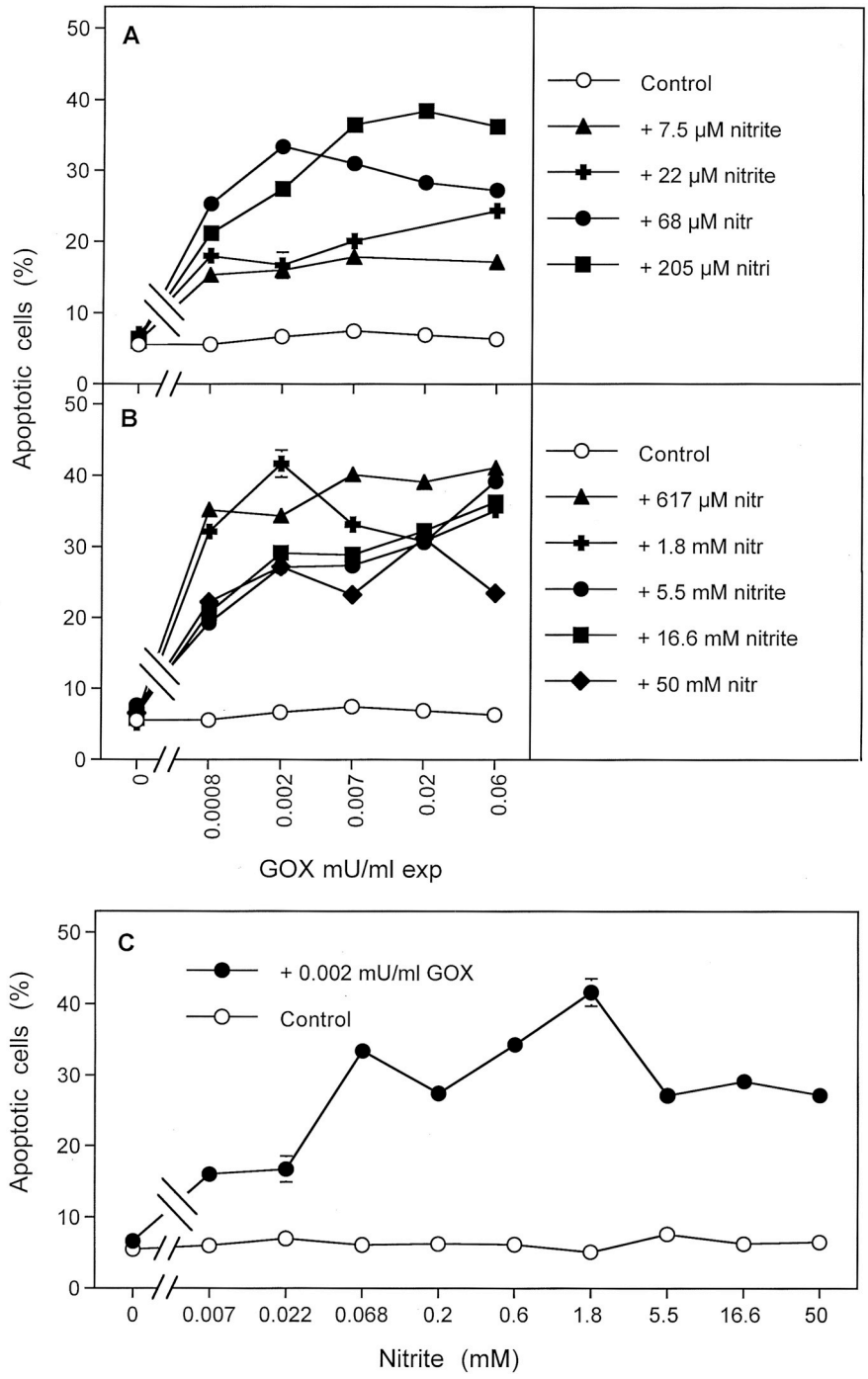


Fig. 3. Dependency of the synergistic effect on the concentrations of GOX and nitrite|. MKN-45 cells were treated with the indicated concentrations of GOX and nitrite for 2.5 h and then the percentages of apoptotic cells were determined.
The results show that the combination of GOX and nitrite was required to obtain apoptosis induction in the dose range chosen for the two compounds. They also show that the dependency of apoptosis induction on the concentration of GOX was sharper than that obtained for nitrite.
Statistical analysis: The synergistic effects mediated by GOX and nitrite were highly significant ($p < 0.001$) at all concentrations of GOX and nitrite.

2.6. Knockdown by treatment with specific small interfering ribonucleic acids (siRNAs)

SiRNAs were obtained from Qiagen (Hilden, Germany).

The following siRNAs were used:

Control siRNA which does not affect any known target in human and murine cells (siCo):

sense: r(UUCUCCGAACGUGUCACGU)dTdT.

antisense: CGUGACACGUUCGGAGAA)dTdT;

2.7. SiRNAs directed towards human NADPH oxidase-1 (NOX1)

custom-made siRNA directed towards NADPH oxidase-1 variant a (**siNOX1-a)**: target sequence: CCG ACA AAT ACT ACT ACA CAA.

sense: r(GAC AAA UAC UAC UAC ACA A)dTdT.

antisense: r(UUG UGU AGU AGU AUU UGU C)dGdG;

SiRNAs were dissolved in suspension buffer supplied by Qiagen at a concentration of 20 µM. Suspensions were heated at 90 °C for 1 min, followed by incubation at 37 °C for 60 min. Aliquots were stored at −20 °C.

Before transfection, 88 µl of medium without serum and without antibiotics were mixed with 12 µl Hyperfect solution (Qiagen) and the required volume of specific siRNA or control siRNA to reach the desired concentration of siRNA during transfection (the standard concentration of siRNA was 10 nM for 208Fsrc3 cells). The mixture was treated by a Vortex mixer for a few seconds and then allowed to sit for 10 min. It was then gently and slowly added to 200 000 208Fsrc3 cells/well in 2.3 ml medium supplemented with 5% FBS and antibiotics (6-well

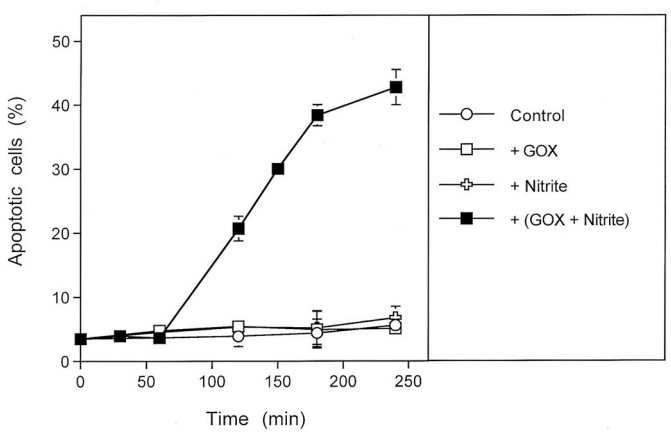


Fig. 4. Kinetics of apoptosis induction mediated through the synergistic effect between GOX and nitrite.
MKN-45 cells remained either untreated (control), or received either 0.05 mU/ml GOX or 1 mM nitrite, as well as a combination of GOX and nitrite. The percentages of apoptotic cells were determined kinetically.
The results show that neither GOX nor nitrite caused apoptosis induction when applied alone. The kinetics of apoptosis induction in the presence of GOX and nitrite was characterized by a lag phase of about 1 h, followed by constant increase in apoptotic cells. Finally, the kinetics approached a plateau phase.
Statistical analysis: Apoptosis induction mediated by GOX and nitrite determined all time points after 60 min was highly significant ($p < 0.001$), whereas there was no significant apoptosis induction when GOX or nitrite were applied alone.

plates). The cells were incubated at 37 °C in 5% CO_2 for 24 h. Transfected cells were centrifuged and resuspended in fresh medium at the required density before use.

Determination of the efficiency of siRNA-mediated knockdown. The siRNA transfection system as described above had been optimized to allow a reproducible transfection efficiency of more than 95% of the cells and to avoid toxic effects [Bauer, unpublished data).

The efficiency of knockdown by siNOX1 was based on determination of relative superoxide anion concentrations through a functional SOD-dependent quantitative assay [62] and was more than 90%.

2.8. Statistics

Statistical analysis. In all experiments, assays were performed in duplicate and empirical standard deviation were calculated. Absence of standard deviation bars indicates that the standard deviation was too small to be reported by the graphic program. Empirical standard deviations merely demonstrate reproducibility in parallel assays but do not allow statistical analysis of variance. The experiments have been repeated at least twice (with duplicate assays). The Yates continuity corrected chi-square test was used for the statistical determination of significances ($p < 0.01$ = significant; $p < 0.001$ = highly significant).

3. Results

3.1. H_2O_2 and nitrite interact synergistically in the induction of apoptosis of tumor cells

For the evaluation of potential synergistic effects between H_2O_2 and nitrite or nitrate during apoptosis induction in malignant cells, increasing concentrations of H_2O_2 were added to human MKN-45 gastric carcinoma cells. The experiments were performed in the absence or presence of 1 mM nitrite or nitrate, respectively. Where indicated, assays also received either the singlet oxygen scavenger histidine or the peroxynitrite decomposition catalyst FeTPPS. As shown in Fig. 1, the tumor cells were resistant to H_2O_2 up to a concentration of 160 μM. Higher concentrations of H_2O_2 caused apoptosis induction in a concentration-dependent mode. Apoptosis induction by high concentrations of H_2O_2 seemed not to be due to direct H_2O_2-dependent apoptosis induction. It rather seemed to be mediated by singlet oxygen-dependent step, as it was abrogated by the singlet oxygen scavenger histidine. This finding is in line with the recently determined complex interaction between relatively high concentrations of H_2O_2 and tumor cells, which is based on H_2O_2-dependent inhibition of SOD, followed by superoxide anion-dependent inhibition of catalase, generation of secondary singlet oxygen, inactivation of protective catalase and reactivation of intercellular apoptosis-inducing ROS/RNS signaling [47].

An initial concept for the action of CAP and PAM had been based on the concept of triggering effects of CAP/PAM-derived species and subsequent autoamplificatory reactions specifically of tumor cells [1,46,76].

When nitrite was added together with H_2O_2, a substantial synergistic effect on apoptosis induction was observed, leading to apoptosis induction in the presence of rather low concentrations of H_2O_2 (Fig. 1). The synergistic effect between nitrite and H_2O_2 was dependent on singlet oxygen generation and required the involvement of peroxynitrite as an intermediate, as seen from the inhibitor profile. In contrast to nitrite, nitrate caused no synergistic effect together with H_2O_2. Neither nitrite nor nitrate caused detectable apoptosis-inducing effect when they were applied in the absence of H_2O_2.

When H_2O_2 was generated steadily by the H_2O_2 generator glucose oxidase (GOX), the same effects on tumor cells were observed as seen before for bolus addition of H_2O_2 (Fig. 2). High concentrations of GOX caused singlet oxygen-dependent apoptosis induction, in analogy to the findings obtained with high concentrations of directly added H_2O_2, as shown in the previous Fig. 1. Lower concentrations of GOX synergized with nitrite in singlet oxygen- and peroxynitrite-dependent apoptosis induction. The singlet oxygen-dependent step seemed to be nearly completed within the first 20 min after addition of GOX to assays containing nitrite. As GOX generates a constant flux of H_2O_2 and allows to establish steady state conditions, whereas bolus addition of H_2O_2 has the eminent problem of varying concentrations during addition, the next experiments in this study were performed with GOX H_2O_2 source.

The synergistic effect between H_2O_2 and nitrite was dependent on the concentrations of the H_2O_2 generator GOX and on the concentration of nitrite (Fig. 3). Thereby the concentration of the H_2O_2 generator seemed to have a stronger determining effect than the concentration of nitrite.

3.2. The kinetic analysis of apoptosis induction

Kinetic analysis confirmed that neither H_2O_2 nor nitrite alone induced apoptosis in MKN-45 cells at the concentrations studied, whereas their combination resulted in efficient synergistic apoptosis induction (Fig. 4). Induction of apoptosis was characterized by an initial lag phase of 60 min, followed by a linear increase in the percentage of apoptotic cells with time.

This characteristic and reproducible kinetics was instrumental to define the role of specific ROS/RNS at defined steps of this scenario. The singlet oxygen scavenger histidine, the peroxynitrite decomposition catalyst FeTPPS, as well as caspase-8 inhibitor completely prevented apoptosis induction by H_2O_2 and nitrite, provided these inhibitors had been added prior to GOX and nitrite (Fig. 5). Addition of the inhibitors 30 min after addition of GOX and nitrite, or later, did no longer allow inhibition of apoptosis. This finding points to a very early singlet oxygen-, peroxynitrite- and caspase-8-dependent step. This step seems to be completed already at 30 min after addition of GOX and nitrite. The results obtained with the aquaporin inhibitor Ag^+ indicated that aquaporins also seem to play a dominant role relatively early in

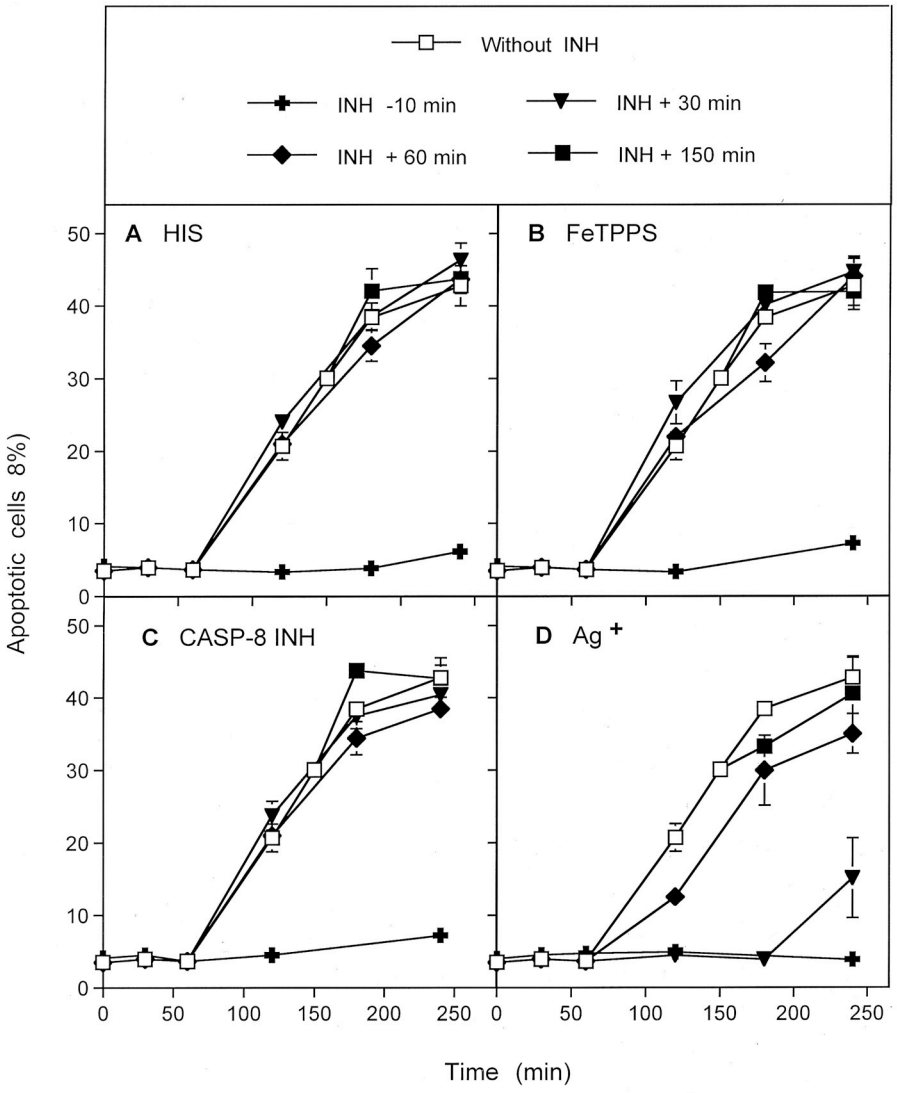


Fig. 5. Early signaling effects in GOX/nitrite-mediated apoptosisi induction in tumor cells.
MKN-45 cells were treated with 0.05 mU/ml GOX plus 1 mM nitrite in the absence or presence of the singlet oxygen scavenger histidine (HIS) (2 mM), the peroxynitrite decomposition catalyst FeTPPS (25 μM), caspase-8 inhibitor (25 μM) or the aquaporin inhibitor Ag^+ (5 μM). The inhibitors had either been added 10 min before GOX/nitrite, or 30 min, 60 min or 150 min after their addition. Apoptosis induction was determined kinetically.The results show that histidine, FeTPPS and caspase-8 inhibitor blocked an early GOX/nitrite-mediated process that seems to be completed within 30 min. The process inhibited by Ag^+ also seems to occur early, but required about 1 h to be nearly completed.
Statistical analysis: Inhibition of GOX/nitrite-mediated apoptosis induction by histidine, FeTPPS and caspase-8 inhibitor was highly significant ($p < 0.001$), when the inhibitors had been added prior to GOX and nitrite, whereas there was no significant inhibition when the inhibitors had been added later. Inhibition of GOX/nitrite-mediated apoptosis induction by Ag^+ was highly significant ($p < 0.001$), when the inhibitor had been added 10 min before or 30 min after GOX and nitrite, but not when it had been added later.

apoptosis induction. The aquaporin-dependent process seemed to be nearly completed at 60 min after addition of GOX and nitrite. It thus was clearly different from the singlet oxygen-dependent initial process and seemed to extend into the beginning of the longer-lasting second step of apoptosis induction.

A completely different inhibition profile was seen for the NOX1 inhibitor AEBSF, the HOCl scavenger taurine, the hydroxyl radical scavenger mannitol and caspase-9 inhibitor. This group of inhibitors still completely blocked apoptosis induction, when they had been added as late as 60 min after addition of GOX and nitrite (Fig. 6). Even when the kinetics of apoptosis induction had reached the point of 30% apoptotic cells at 150 min, addition of these inhibitors still caused an immediate block of further apoptosis induction. These findings indicated that this group of inhibitors target ROS/RNS that are actually involved during the late and longer-lasting stage of apoptosis induction through intercellular HOCl signaling.

In line with formal logistics, inhibitors that acted at an early stage, but not at a late one, pointed clearly to an involvement of their target in an early process. However, it was not possible to clarify from this approach alone whether the inhibitors that acted at the late stage of the process also had an inhibitory function during the early stage or not. Therefore, completion of the picture required further analysis that involved the dissection of the process into discrete steps.

3.3. Dissection of H_2O_2/nitrite-mediated apoptosis induction into mechanistically defined steps: the concept

Completed studies on the control of intercellular ROS/RNS signaling by membrane-associated catalase and its reactivation through singlet oxygen-dependent processes [47,50,57,74] have shown that reactivation of NOX-driven apoptosis-inducing intercellular ROS/RNS signaling requires the preceding inactivation of membrane-associated catalase of tumor cells. It is then followed by intercellular ROS/RNS signaling, ending with hydroxyl radical-mediated lipid peroxidation and the onset of the mitochondrial pathway of apoptosis [47,50,57,63,64,74]. Therefore it seemed obvious that the short, initial singlet oxygen-dependent step of nitrite/H_2O_2 action towards tumor cells, as seen in the preceding kinetic analysis, might reflect inactivation of tumor cell protective catalase. The longer-lasting subsequent step might then comprise intercellular ROS/RNS signaling and the execution of the mitochondrial pathway of apoptosis. The intermediate aquaporin-mediated step might be related to the control of the intracellular glutathione level through influx of extracellular H_2O_2 that is present in abundance after catalase inhibition. The analysis of these three steps, i.e. inactivation of catalase, aquaporine-mediated influx of H_2O_2 and intercellular apoptosis-inducing signaling is outlined in the next subchapters. This approach allowed to define the molecular species involved in each single step and to finally propose a model of the

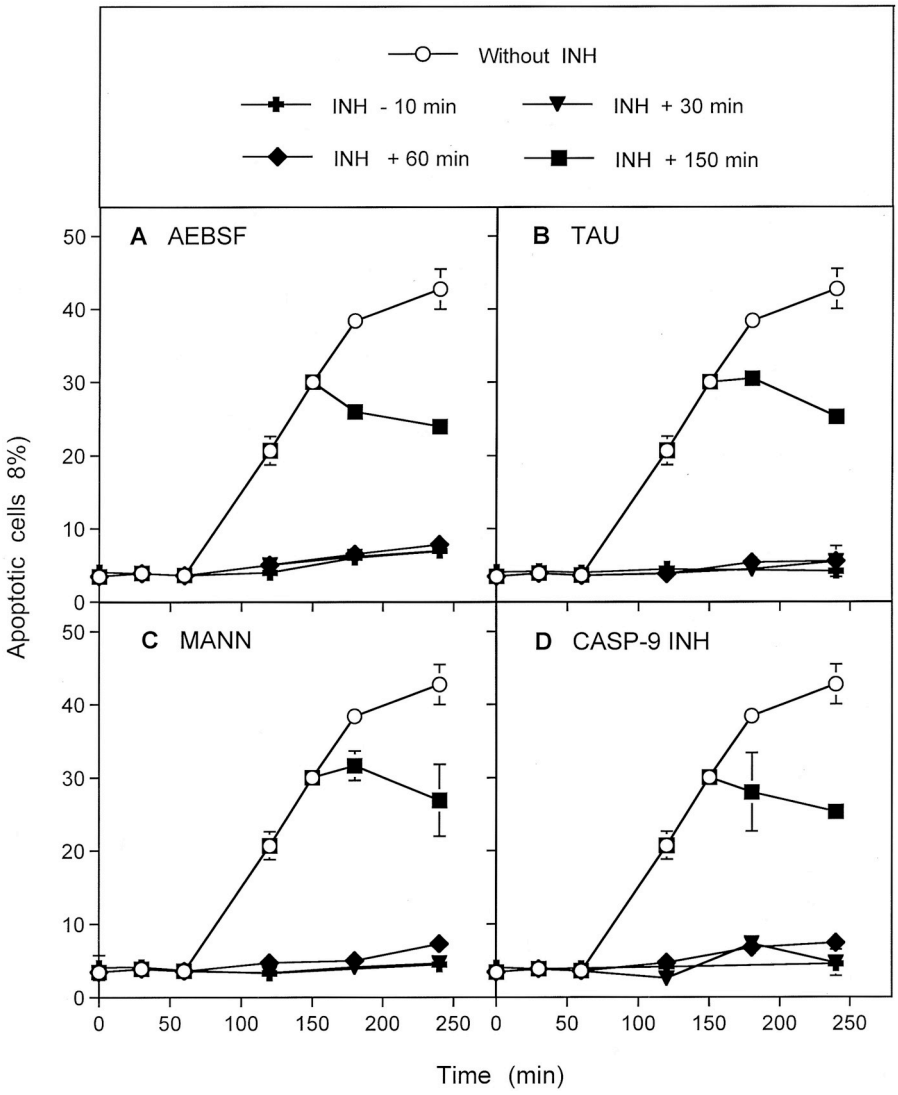


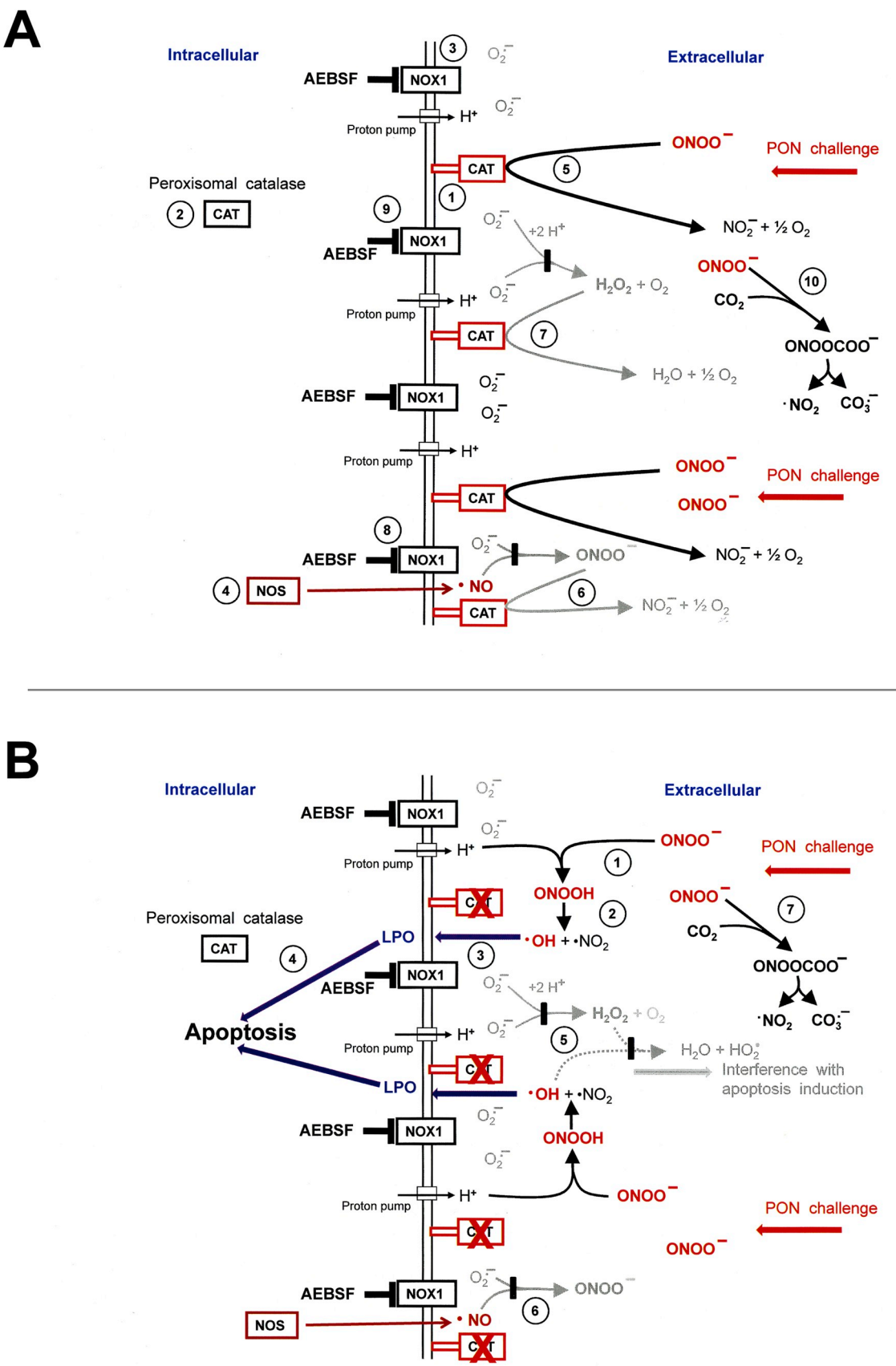
Fig. 6. Late signaling effects in GOX/nitrite-mediated apoptosis induction in tumor cells.
MKN-45 cells were treated with 0.05 mU/ml GOX plus 1 mM nitrite in the absence or presence of the NOX1 inhibitor AEBSF (100 μM), the HOCl scavenger taurine (50 mM), the hydroxyl radical scavenger mannitol (20 mM) or caspase-9 inhibitor (25 μM). The inhibitors had either been added 10 min before GOX/nitrite, or 30 min, 60 min or 150 min after their addition. Apoptosis induction was determined kinetically.The result show that all four inhibitors blocked a late signaling effect, most likely HOCl signaling and subsequent mitochondrial pathway of apoptosis. Even if the inhibitors had been added at 150 min, they still caused immediate block of apoptosis induction. The slight decrease of the percentage of apoptotic cells after inhibitor addition at 150 min is due to fragility of apoptotic cells as well as phagocytosis by still surviving neighbouring cells. Statistical analysis: Inhibition of GOX/nitrite-mediated apoptosis induction by all four inhibitors was highly significant ($p < 0.001$), when the inhibitors had been added 10 min before or 30 or 60 min after GOX/nitrite. Addition of the inhibitors 150 min after GOX and nitrite caused significant inhibition ($p < 0.01$).

intercalated action of these steps.

3.3.1. Analysis of initial inactivation of membrane-associated catalase by H_2O_2 and nitrite

Challenging of tumor cells with exogenous peroxynitrite allows to specifically determine their degree of protection by membrane-associated catalase in a quantitative way (Fig. 7). Any variation of intracellular catalase does not contribute to the outcome of this test, as peroxynitrite cannot pass the cell membrane without causing lipid peroxidation and therefore its effective concentration cannot be modulated by intracellular catalase. As shown in Fig. 8 A, the catalase inhibitor 3-aminotriazole, our positive control, caused sensitization of tumor cells for apoptosis induction through an exogenous peroxynitrite challenge. Pretreatment of the tumor cells with either GOX or nitrite did not cause sensitization for the peroxynitrite challenge above the level of untreated control cells (Fig. 8 B). However, pretreatment with the combination of GOX and nitrite sensitized the cells to the same degree as 3-AT. Sensitization by GOX plus nitrite was completely prevented when the singlet oxygen scavenger histidine had been present during pretreatment. These findings allow to conclude that pretreatment with H_2O_2 plus nitrite caused singlet oxygen-dependent inactivation of membrane-associated catalase. A central and necessary technical control shown in Fig. 8C ensured that the apoptosis-inducing effect of the peroxynitrite challenge was indeed due to peroxynitrite action, as addition of either the peroxynitrite decomposition catalyst FeTPPS or of catalase, immediately before the peroxynitrite challenge, caused complete abrogation of apoptosis induction. The inhibitory effect of the hydroxyl radical scavenger during the challenge step ensured that peroxynitrite action depended on hydroxyl radicals. This is in line with the formation of peroxynitrous acid in the vicinity of membrane-associated proton pumps [50] and subsequent spontaneous decomposition of peroxynitrous acid into hydroxyl radials and NO_2.

Ensured through these initial controls, the test system based on peroxynitrite challenge was therefore valid for determination of the nature of ROS/RNS involved in the inactivation of membrane-associated catalase after treatment with GOX and nitrite. Pretreatment with GOX and nitrite was performed in the absence or presence of the inhibitors and scavengers shown in Fig. 9 A-C. After pretreatment, the cells were washed and challenged with exogenous peroxynitrite. Fig. 9 A, B demonstrates that inactivation of membrane-associated catalase by GOX and nitrite seemed to require the concerted interaction between NOX-derived superoxide anions, H_2O_2, NOS-derived NO, peroxynitrite, singlet oxygen, hydroxyl radicals and caspase-8, as it was inhibited by AEBSF, EUK-134, L-NAME, FeTPPS, histidine, mannitol and caspase-8 inhibitor. The inhibitory effects of AEBSF and L-NAME pointed to a determining role of NOX1 and NOS in this biochemical process. Inactivation of catalase did not require the presence of HOCl or the activity of peroxidase, as taurine and ABH caused no inhibitory effect (Fig. 9 B). As outlined under Discussion, the knowledge of the species involved in catalase inactivation allowed to draw a reaction scheme for



(caption on next page)

Fig. 7. Determination of the inactivation of membrane-associated catalase through a peroxynitrite challenge: the principle
A. Protection of tumor cells towards exogenous peroxynitrite
Tumor cells are characterized by expression of membrane-associated catalase (#1). In addition, they possess intracellular (peroxisomal) catalase (#2). Due to their malignant state, tumor cells express membrane-associated NADPH oxidase-1 (NOX1) that generates extracellular superoxide anions (#3). Intracellular NO synthase (NOS) generates NO that passes through the membrane (#4). Membrane-associated catalase decomposes exogenously added peroxynitrite ["peroxynitrite challenge"] (#5), peroxnitrite generated by the tumor cells (#6), and H_2O_2 derived from dismutation of NOX1-derived superoxide anions (#7). The addition of AEBSF immediately before the peroxynitrite challenge prevents the formation of cell-derived peroxynitrite and of cell-derived H_2O_2. The presence of AEBSF thus allows to focus on the effects induced specifically by exogenous peroxynitrite at defined concentrations and in addition prevents the side reaction between peroxynitrite and H_2O_2. Distant of the membrane a certain concentration of peroxnitrite is lost through reaction with CO_2 (#10).
B. Sensitization of tumor cells for peroxynitrite action after inactivation of their membrane-associated catalase.
When membrane-associated catalase has been inactivated in a step preceding the peroxynitrite challenge, exogenous peroxnitrite is no longer decomposed and has a chance to get protonated by membrane-associated proton pumps (#1). The resultant peroxynitrous acide readily decomposes into NO_2 and hydroxyl radicals (#2). Hydroxyl radicals cause lipid peroxidation (LPO) (#3), which triggers apoptosis induction through the mitochondrial pathway of apoptosis (#4). The presence of the NOX inhibitor AEBSF during the PON challenge prevents consumption of hydroxyl radicals by H_2O_2 (#5) as well as generation of cell-derived peroxynitrite (#6). In contrast to membrane-associated catalase, intracellular catalase does not affect apoptosis induction by exogenous peroxynitrite, as it cannot reach the substrate.

the processes that starts with the interaction between H_2O_2 and nitrite and seems to be executed by singlet oxygen. The whole process is completed within less than 30 min.

The validity of these conclusions was dependent on the ensurance that inhibitors that were present during preincubation of the cells were completely removed during the washing steps, and therefore could not interfere with the peroxynitrite challenge later on. As FeTPPS acts catalytically, it represents the most effective and therefore potentially problematic tool in this respect. As shown in Fig. 10 A, FeTPPS added after preincubation of the cells, but before the washing step, only had a minor negative effect on the subsequent challenging step by peroxynitrite. Therefore, the washing step was proven to be sufficient to remove the inhibitor from the test system. Any substantial interference of inhibitors used during preincubation with the challenging step is therefore excluded through the washing step, provided the inhibitors act in a reversible mode.

Differential addition of caspase inhibitors was used to dissect the role of caspases during enhancement of signaling and for the mediation of cell death by the PON challenge. As shown in Fig. 10 B, the presence of caspase-8 inhibitor during pretreatment of the cells with GOX and nitrite prevented catalase inactivation, but its presence during and after the PON challenge did not interfere with apoptosis induction. In contrast, addition of caspase-9 inhibitor immediately before the PON challenge prevented subsequent apoptosis induction. These findings allow to dissect the effects of caspase-8 and caspase-9. The action of caspase-8 seems to be specifically related to the control of early signaling events involved in catalase inactivation, but not directly to execution of apoptosis. In contrast,caspase-9 seems to mediate the mitochondrial pathway triggered by PON.

3.3.2. Analysis of reactivated intercellular apoptosis-inducing signaling after initial inactivation of membrane-associated catalase by H_2O_2 and nitrite

The experimental confirmation of inactivation of membrane-associated catalase by a short preincubation with GOX and nitrite, as shown in chapter 3.3.1 was the basis for a subsequent study of the mechanistic details of intercellular signaling events that are following catalase inactivation. For this purpose, tumor cells were pretreated with GOX and nitrite for 25 min, washed and then subjected to further incubation in the absence or presence of selected relevant inhibitors. As shown in Fig. 11 A, apoptosis induction required pretreatment with GOX and nitrite before the washing step. Treatment with either GOX or nitrite alone was without significant effects. Apoptosis-inducing signaling after pretreatment with GOX and nitrite required superoxide anions, H_2O_2, peroxidase, HOCl, hydroxyl radicals, aquaporins, caspase-9 and caspase-3, as determined from the inhibitor profile This is indicative for the preferential establishment of the HOCl signaling pathway followed by the mitochondrial pathway of apoptosis [44,63]. Aquaporins also seem to be absolutely essential for this step, as silver ions caused complete inhibition of apoptosis. In contrast, singlet oxygen, NO, peroxynitrite and caspase-8 did not seem to contribute to apoptosis-inducing signaling after pretreatment of MKN-45 tumor cells with GOX and nitrite. The strong and selective reactivation of the HOCl signaling pathway points to a rather efficient inactivation of catalase, as HOCl synthesis requires the presence of substantial concentrations of H_2O_2 [42].

The presence of substantial concentrations of H_2O_2 and HOCl during HOCl signaling exert a strong negative impact on NO/peroxynitrite signaling [45]. This explains the lack of NO/peroxynitrite signaling in the experiment described in Fig. 11 A. When, however, the concentration of NO was increased through the addition of an exogenous NO donor to GOX/nitrite-pretreated cells, HOCl signaling seemed to be completely suppressed and NO/peroxynitrite signaling was now prominent as the dominating cause for apoptosis-inducing signaling (Fig. 11 B). NO/peroxynitrite signaling required superoxide anions, peroxynitrite and hydroxyl radicals. As the required NO was derived from the NO donor, inhibition of NOS by L-NAME had no inhibitory effect. High concentrations of catalase (1000 U/ml) were required for inhibition of NO/peroxynitrite signaling. Like HOCl signaling, NO/peroxynitrite signaling was under tight control by aquaporins and was mediated through the mitochondrial pathway of apoptosis, utilizing caspases-9 and -3. It was independent of caspase-8. Suppression of HOCl signaling in the presence of the NO donor was seen by the lack of inhibition through taurine, ABH and EUK-134.

As GOX and nitrite had been removed before the analysis shown in Fig. 11 A and B, all molecular species (except NO) involved in the signaling processes studied must have been derived from the tumor cells or generated through interaction between tumor cell derived species.

3.3.3. The role of aquaporins for apoptosis induction mediated by H_2O_2 and nitrite

The kinetic analysis of apoptosis induction after combined action of GOX and nitrite had shown that aquaporins seem to play a dominant role in the time window after singlet oxygen-dependent inactivation of protective catalase and before execution of intercellular ROS/RNS-dependent apoptosis-inducing signaling (please see Fig. 6). This finding led to the working hypothesis that aquaporine-dependent influx of H_2O_2 after local inactivation of protective catalase might have an effect on the intracellular glutathione level. This might then interfere with a glutathione/glutathione peroxidase-4-dependent counteraction towards apoptosis induction triggered by hydroxyl radical-mediated lipid peroxidation [77]. To address this question, the glutathione level in tumor cells was lowered by either inhibiting glutathione synthase through BSO [78] or inhibiting the cysteine-glutamate transporter sulfasalazine [79]. Tumor cells were pretreated for 14 h in the presence of 50 μM BSO, or 100 μM sulfasalazine, or without additions. They were then treated with GOX, nitrite or a combination of both, in the presence or absence of the aquaporin inhibitor Ag^+(5μM). As shown in Fig. 12, apoptosis induction mediated by the combination of GOX and nitrite in

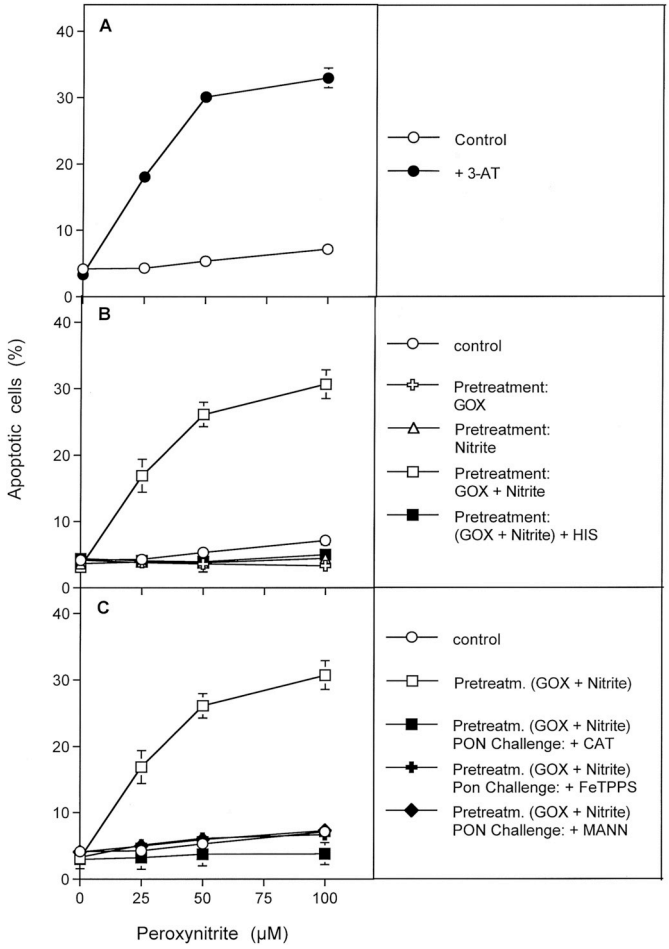


Fig. 8. Treatment of tumor cells with GOX/nitrite causes inactivation of membrane-associated catalase.

A: MKN-45 cells remained untreated or received 25 mM 3-AT;

B: MKN-45 cells remained untreated or where treated with 0.05 mU/ml GOX, or 1 mM nitrite, 0.05 mU/ml GOX +1 mM nitrite, or GOX/nitrite plus 2 mM histidine for 25 min. The cells were then subjected to three washing cycles and resuspended in fresh medium.

Cells treated according to A and B received 100 μM AEBSF and were then challenged with the indicated concentrations of peroxynitrite. The percentage of apoptotic cells were determined after 2 h .

C: MKN-45 cells remained untreated or were pretreated with 0.05 mU/ml GOX plus 1 mM nitrite for 25 min. The cells were subjected to three cycles of washing and were then resuspended in fresh medium, containing 100 μM AEBSF. Some assays that had been pretreated with GOX/nitrite received either 100 U/ml catalase, or 25 μM FeTPPS or 20 mM mannitol before the peroxynitrite challenge. All assays were challenged with the indicated concentrations of peroxnitrite. The percentages of apoptotic cells were determined after 2 h.

These results show that pretreatment with GOX plus nitrite, but not with GOX or nitrite alone, caused the same degree of catalase inactivation as application of the catalase inhibitor 3-AT. Catalase inactivation by GOX/nitrite seemed to be mediated by singlet oxygen. Part C ensures that apoptosis induction through a challenge with exogenous peroxynitrite was indeed due to the action of peroxynitrite, as it was prevented through the presence of catalase, FeTPPS or mannitol during the challenge. Catalase and FeTPPS decompose peroxynitrite and mannitol scavenges hydroxyl radicals that are released from peroxynitrous acid [42].

Statistical analysis: Apoptosis induction in the presence of peroxynitrite and either 3-AT or GOX/nitrite was highly significant ($p < 0.001$), whereas there was no significant apoptosis induction by GOX or nitrite applied alone. The dependence of apoptosis induction on the concentrations of peroxynitrite applied was highly significant ($p < 0.001$) for the difference between no peroxynitrite/25 μM peroxynitrite and 25 μM peroxynitrite/50 μM peroxynitrite (in the presence of either 3-AT or GOX/nitrite), whereas the difference between 50 μM and 100 μM peroxynitrite was not significant.All inhibitors used in this experiment caused highly significant ($p < 0.001$) inhibition of peroxynitrite-mediated apoptosis induction after pretreatment of the cells with GOX/nitrite.

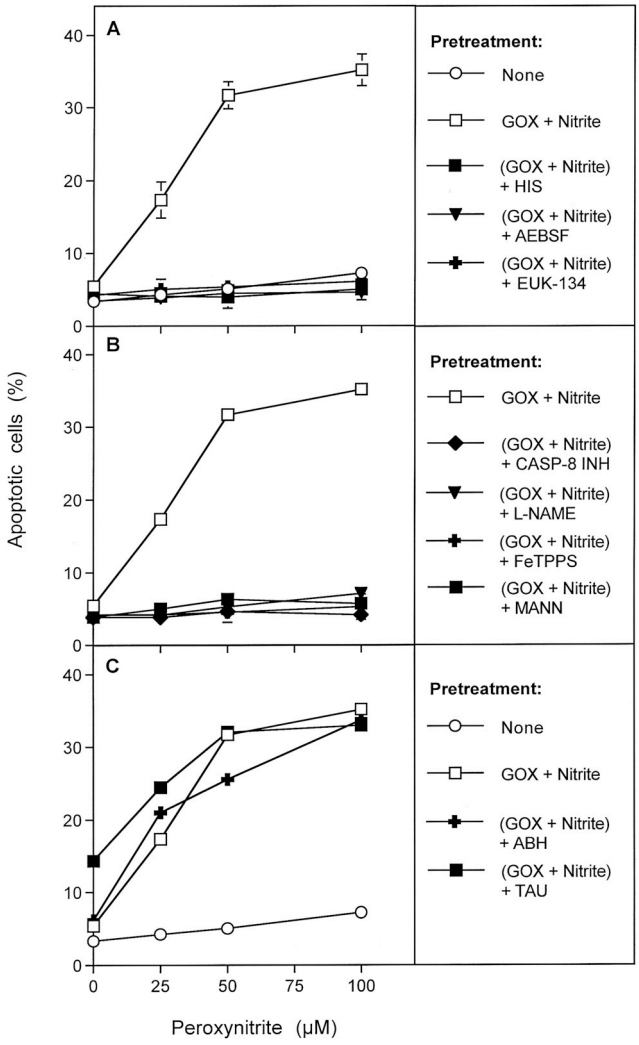


Fig. 9. Biochemical mechanism of catalase inactivation by GOX/nitrite.

MKN-45 cells were not pretreated or were pretreated with 0.05 mU/ml GOX plus 1 mM nitrite without further inhibitors or in the presence of 2 mM of the singlet oxygen scavenger histidine, 100 μM of the NOX1 inhibitor AEBSF,. 25 μM of the catalase mimetic EUK-134, 25 μM caspase-8 inhibitor, 2.4 mM of the NOS inhibitor L-NAME, 25 μM of the peroxynitrite decomposition catalyst FeTPPS, 20 mM of the hydroxyl radical scavenger mannitol, 150 μM of the peroxidase inhibitor ABH, 50 mM of the HOCl scavenger taurine. After preincubation for 25 min, all assays were washed three times and resuspended in fresh medium plus 100 μM AEBSF. Peroxynitrite was added at the indicated concentrations. The percentages of apoptotic cells were determined after 2 h.

The results confirm that pretreatment of tumor cells with GOX/nitrite inactivated membrane-associated catalase through a biochemical mechanism that seemed to involve singlet oxygen, NOX1-derived superoxide anions, H_2O_2, caspase-8, NOS-derived NO, peroxynitrite and hydroxyl radicals, whereas it was independent of peroxidase activity and HOCl.

Statistical analysis: Apoptosis induction mediated by peroxynitrite in GOX/nitrite-pretreated cells was highly significant ($p < 0.001$). The presence of histidine, AEBSF, EUK-134, caspase-8 inhibitor, L-NAME, FeTPPS, or mannitol caused highly significant inhibition of apoptosis induction ($p < 0.001$), whereas the presence of taurine or ABH during pretreatment caused no significant inhibitory effect.

control tumor cells started after a lag phase of 1 h and was completely blocked by the aquaporin inhibitor. In line with previous results, application of either GOX or nitrite alone did not cause apoptosis induction in these cells. Cells pretreated with either BSO (Fig. 12 B) or sulfasalazine (Fig. 12C) responded to the combination of GOX and nitrite without the lag phase that was characteristic for the reaction of control

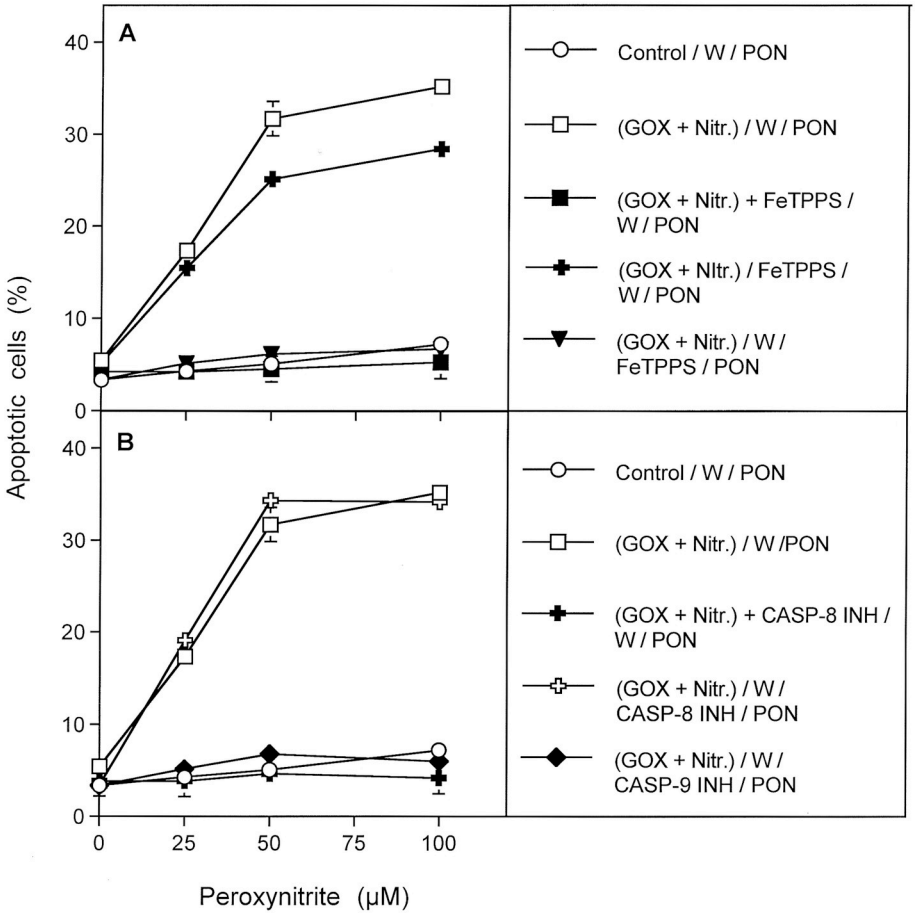


Fig. 10. Control assays related to catalase inactivation by GOX/nitrite.

A. MKN-45 cells remained either untreated (control, open circle) or were pretreated with 0.05 mU/ml GOX plus 1 mM nitrite for 25 min (open square), before they were subjected to three cycles of washing, resuspended in fresh medium + 100 μM AEBSF, and challenged with the indicated concentrations of peroxynitrite. In addition, assays were pretreated with GOX/nitrite in the presence of 25 μM FeTPPS (closed square, "(GOX + Nitr.) + FeTPPS/W/PON)"), or pretreated for 25 min with GOX/nitrite, and then FeTPPS was added before the washing step (closed crosses, (GOX + Nitr.)/FeTPPS/W/PON)), or FeTPPS was added to cells that had been pretreated with GOX/nitrite for 25 min and had been washed, immediately before the PON challenge (closed triangles; (GOX + Nitr.)/W/FeTPPS/PON. The percentages of apoptotic cells were determined after 2 h. The results confirm that treatment with GOX/nitrite caused inactivation of membrane-associated catalase of tumor cells. The presence of the peroxynitrite decomposition catalyst FeTPPS during GOX/nitrite pretreatment prevented inactivation of catalase, as it prevented the generation of singlet oxygen. The presence of FeTPPS during the peroxynitrite challenge prevented apoptosis induction, as it decomposed the challenging peroxynitrite. FeTPPS added after preincubation, but before washing only had a minor effect on apoptosis induction. This demonstrates that the washing step was efficiently removing the scavenger.

B. Part B was performed in an analogous mode as part A, but was focusing on the role of caspases for the inactivation of catalase.

Inactivation of catalase by pretreatment with GOX/nitrite was prevented when caspase-8 inhibitor (25 μM) had been present during pretreatment (closed crosses, (GOX + Nitr.) + CASP-8 INH/W/PON), whereas caspase-8 inhibitor was without effect when it was added after preincubation with GOX/nitrite, but before the peroxynitrite challenge (open crosses). The addition of 25 μM caspase-9 inhibitor after preincubation, but before the peroxynitrite challenge, completely prevented apoptosis induction (closed diamonds).

These data confirm that caspase-8 played a role during inactivation of catalase by GOX/nitrite, but not for peroxynitrite-mediated cell death, whereas caspase-9 was involved in cell death through the mitochondrial pathway of apoptosis.

Statistical analysis: A: The presence of FeTPPS either during pretreatment of the cells with GOX/nitrite or during the peroxynitrite challenge caused highly significant inhibition of apoptosis ($p < 0.001$), whereas the application of FeTPPS immediately before the washing step that followed preincubation with GOX/nitrite did not cause significant inhibition. B:Application of caspase-8 inhibitor during pretreatment with GOX/nitrite caused highly significant inhibition of apoptosis induction ($p < 0.001$), whereas its application immediately before the peroxynitrite challenge did not cause significant inhibition. In contrast, the presence of caspase-9 inhibitor during the peroxxynitrite challenge caused significant inhibition.

cells. In contrast to the reaction of control cells, apoptosis induction in BSO- or sulfasalazine-pretreated cells was independent on the action of aquaporins, as shown by the only minor inhibitory effect of the aquaporin inhibitor Ag^+. Apoptosis induction after combined application of GOX and nitrite was dependent on superoxide anions and HOCl in control tumor cells, as well as in tumor cells pretreated with either BSO or sulfasalazine. This was seen by the strong inhibitory effect of AEBSF and taurine for all combinations of treatment, including BSO- or sulfasalazine-pretreated cells in the presence of Ag^+ (Fig. 13). These findings show that apoptosis induction after GOX/nitrite treatment is mediated by the HOCl signaling pathway, irrespective of the intracellular glutathione level. However, in tumor cells with lowered glutathione level, an influx of H_2O_2 through aquaporins seems not to be necessary for the activation of the apoptosis-inducing pathways, in contrast to glutathione-competent control tumor cells. These findings show that aquaporins control a dominant step during apoptosis induction mediated by H_2O_2 and nitrite. This step becomes effective after singlet oxygen-dependent inactivation of membrane-associated catalase and cooperates with the subsequent step of intercellular ROS-dependent apoptosis-inducing signaling.

3.4. Establishment for criteria that allow apoptosis induction by long-lived species from CAP or PAM selectively in tumor cells

Analysis performed previously has shown that nonmalignant cells are more sensitive to apoptosis induction by H_2O_2 than tumor cells, as they lack membrane-associated catalase [42,43]. From these data it is predictable that apoptosis induction in tumor cells through establishment of a synergistic effect between H_2O_2 and nitrite can only be selective for tumor cells when the H_2O_2 concentration is below a concentration that would endanger the survival of nonmalignant cells. The purpose of this chapter is to define the criteria for selective apoptosis induction in tumor cells under consideration of these interactions.

We took advantage of our finding that NOX1 activity controls expression of membrane-associated catalase of tumor cells through H_2O_2, the dismutation product of NOX1-derived superoxide anions [43]. SiRNA-mediated knockdown of NOX1 after 24 h or inhibition of NOX1 over 14 hs therefore also causes reduction of catalase activity below detectability. This is achieved by lack of further catalase expression as well as high turnover of preexisting catalase on the cells [43]. Tumor cells with knockdown of NOX1 or persistent inhibition of NOX1, followed by dramatic decrease of their membrane-associated catalase,

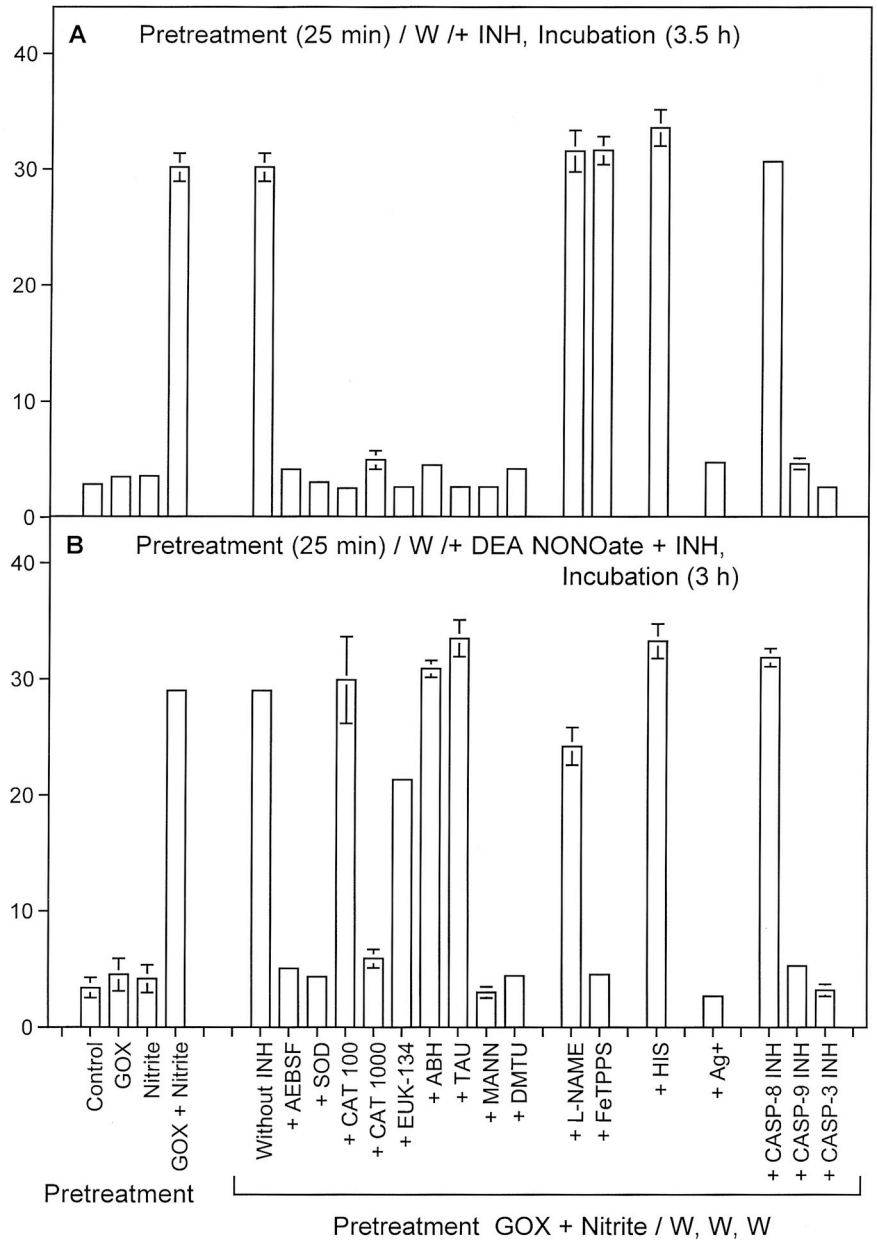


Fig. 11. Pretreatment with GOX/nitrite reactivates intercellular ROS/RNS-mediated apoptosis-inducing signaling

A. MKN-45 cells remained untreated, or where pretreated for 25 min with either 0.05 mU/ml GOX, 1 mM nitrite, or the combination of GOX and nitrite. The assays were subjected to three cycles of washing and resuspended in fresh medium. Cells that had been pretreated with GOX/nitrite remained without inhibitors or received the indicated inhibitors/scavengers. The percentages of apoptotic cells were determined after 3.5 h of incubation.

B. The experiment was performed as described under A. After addition of the inhibitors had been completed, all assays described under B received 0.5 mM of the NO donor DEA NONOate. The percentages of apoptotic cells were determined after 3 h. The concentrations of most inhibitors can be found in the legend to Figure 9, except for SOD (100 U/ml), the hydroxyl radical scavenger dimethyl thiourea (DMTU) (20 mM) and the aquaporin inhibitor Ag^+ (5 μM). Caspase-8 and caspase-9 inhibitors were applied at 25 μM, caspase-3 inhibitor at 50 μM.

The results obtained in part A show that treatment of tumor cells with GOX/nitrite reactivated intercellular apoptosis inducing signaling through the HOCl signaling pathway and executed by caspase-3 and -9, as apoptosis was dependent on superoxide anions, H_2O_2, peroxidase, HOCl, hydroxyl radicals, caspase-9 and caspase-3, whereas NO, peroxynitrite, singlet oxygen and caspase-8 play no role.

In the presence of the NO donor, signaling was shifted to NO/peroxynitrite signaling on the expense of HOCl signaling, as H_2O_2, peroxidase, HOCl were no longer required for apoptosis induction, but signaling was driven by superoxide anions, peroxynitrite and hydroxyl radicals. As NO was supplied by the NO donor, inhibition of NOS was without effect. NO/peroxynitrite-dependent apoptosis inducing signaling did not involve singlet oxygen. It was also independent of caspase-8, but is mediated by caspases-3 and -9.

HOCl-as well as NO/peroxynitrite-dependent apoptosis-inducing signaling were prevented when aquaporin function was inhibited by Ag^+.

Statistical analysis: Pretreatment of tumor cells with GOX/nitrite caused highly significant apoptosis induction ($p < 0.001$) after subsequent incubation.

A: Inhibition of apoptosis induction by all inhibitors/scavengers except L-NAME, FeTPPS, histidine, caspase-8 inhibitor was highly significant ($p < 0.001$).

B: Inhibition of apoptosis induction by all inhibitors/scavengers except 100 U/ml catalase, EUK-134, ABH, taurine, L-NAME, histidine and caspase-8 inhibitor was highly significant ($p < 0.001$).

therefore represent control cells that are genetically identical to the tumor cells, but lack the two essential redox-related elements of tumor cells, i. e. active NOX1 and membrane-associated catalase activity.

Fig. 14 shows that tumor cells pretreated with irrelevant control siRNA showed the pattern to be expected from the previous experiments when they were treated with GOX in the absence or presence of nitrite. There was relatively strong resistance towards apoptosis induction mediated by H_2O_2, but a very prominent synergistic effect between H_2O_2 and nitrite. Both processes were completely prevented when NOX1 was inhibited by AEBSF. In contrast, MKN-45 cells with knockdown of NOX1 ("siNOX1") and subsequent loss of membrane associated catalase activity, were more sensitive to H_2O_2 alone than the corresponding wildtype tumor cells. Furthermore, the cells with NOX1 knockdown did not allow to establish the synergistic effect between H_2O_2 and nitrite. Thus the siNOX-treated tumor cells showed the phenotype of nonmalignant cells, with respect to increased sensitivity for apoptosis induction by H_2O_2 and with lack of response to the synergistic effect of low concentrations of H_2O_2 and nitrite. This allowed to define a window of H_2O_2 concentrations that, together with nitrite caused apoptosis induction in tumor cells, but did not affect the cells with the nonmalignant phenotype. This finding was fully confirmed when MKN-45 cells had been pretreated with the NOX1 inhibitor AEBSF for 14 h and the inhibitor had remained present during the analysis (Fig. 15). Cells treated in this way showed the phenotype of NOX1-negative, catalase-negative control cells and were not affect by GOX below 0.002 mU/ml plus nitrite, whereas the same concentrations of these two compounds established a strong apoptosis-inducing effect on tumor cells.

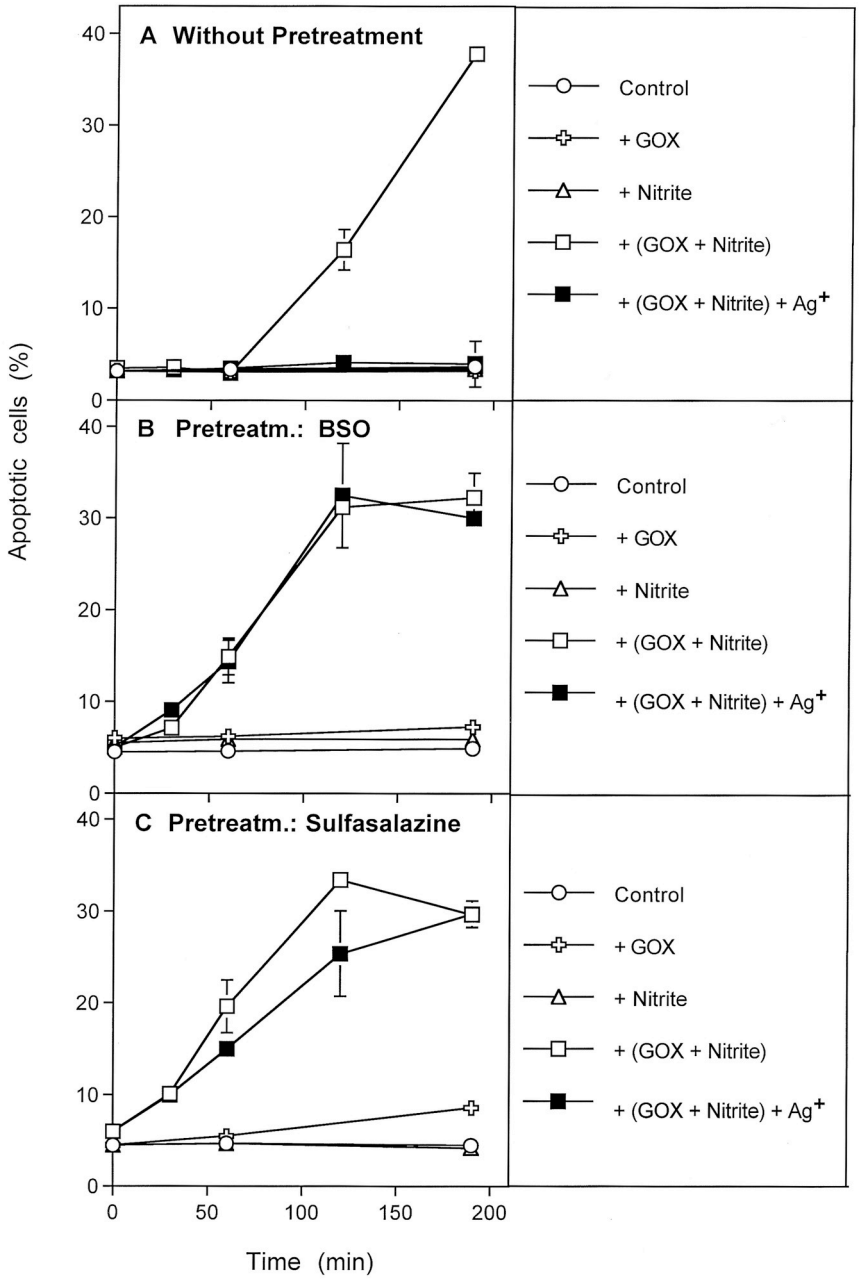


Fig. 12. The role of aquaporins and glutathione for GOX/nitrite-mediated apoptosis induction in tumor cells

MKN-45 cells were incubated for 14 h without an inhibitor (A), or 50 μM BSO, an inhibitor of glutathione synthesis (B), or 100 μM sulfasalazine, an inhibitor of the cysteine-glutamate transporter (C). After preincubation, the cells were washed, resuspended in fresh medium and the compounds were added (GOX: 0.05 mU/ml, nitrite: 1 mM, Ag^+: 5 μM) individually or in combination, as indicated in the legend. Apoptosis induction was determined kinetically.

The results show that the kinetics of apoptosis induction in the presence of GOX and nitrite was characterized by an initial lag phase and was completely inhibited in the presence of the aquaporin inhibitor Ag^+. When the cells had been pretreated with either BSO (B) or with sulfasalazine (C), and thus the intracellular glutathione level had been depleted, the kinetics of apoptosis induction mediated by GOX/nitrite started without a lag phase and seemed to be independent of aquaporin function, as it was not inhibited by Ag^+.

Statistical analysis: A: Apoptosis induction in the presence of GOX/nitrite, as well as inhibition of this process by Ag+ were highly significant ($p < 0.001$). B, C: When cells had been pretreated with BSO (B) or sulfasalazine (C), and subsequently treated with GOX/nitrite, their kinetics of apoptosis induction was shifted leftward in a highly significant way ($p < 0.001$) compared to cells without pretreatment, but analogous treatment with GOX/nitrite (A). There was no significant inhibition of apoptosis induction by Ag^+ in cells pretreated with either BSO or sulfasalazine.

4. Discussion

These data show that the *synergistic effect between nitrite and H_2O_2* is the key for selective apoptosis induction in tumor cells after application of CAP or PAM.

As sole application of nitrite to tumor cells did not cause apoptosis induction, the established interference of nitrite with the catalytic cycle of tumor cell protective catalase [80] is therefore obviously not sufficient to trigger the biochemical steps that lead to apoptosis induction after application of CAP or PAM. As it was confirmed that sole application of H_2O_2 caused apoptosis induction in nonmalignant cells at much lower concentrations than required for tumor cells, the effect of H_2O_2 alone can also not explain selective action of CAP or PAM towards tumor cells. The higher resistance of tumor cells towards exogenous H_2O_2 is explained by the protective function of their membrane-associated catalase [42,43].

Our data and conclusions are in excellent agreement with the previous findings by Kurake et al. and Girard et al. [28,29], who showed that the synergistic effect between PAM-derived H_2O_2 and nitrite is necessary for its selective antitumor effect. Kurake et al., 2016 and Girard et al., 2016 also suggested that peroxynitrite was generated through the interaction between H_2O_2 and nitrite, following the findings by Lukes et al. [33] and in line with the suggestions by Jablonowski and von Woedtke [31]. We extend this scenario by demonstrating that peroxynitrite generated through the interaction between nitrite and H_2O_2 reacts further with residual H_2O_2, leading to the formation of primary singlet oxygen. Extracellular singlet oxygen has been recently shown to possess selective antitumor cell potential, as it can inactivate tumor cell protective catalase, at concentration ranges that are tolerable for nonmalignant cells [57]. We show, that singlet oxygen generated through nitrite/H_2O_2 interaction triggers tumor cells to generate much higher concentrations of secondary singlet oxygen, causing substantial inactivation of tumor cell protective catalase and subsequent reactivation of intercellular apoptosis-inducing ROS signaling. These findings explain how CAP and PAM can induce analogous selective antitumor effects, though PAM only contains long-lived,

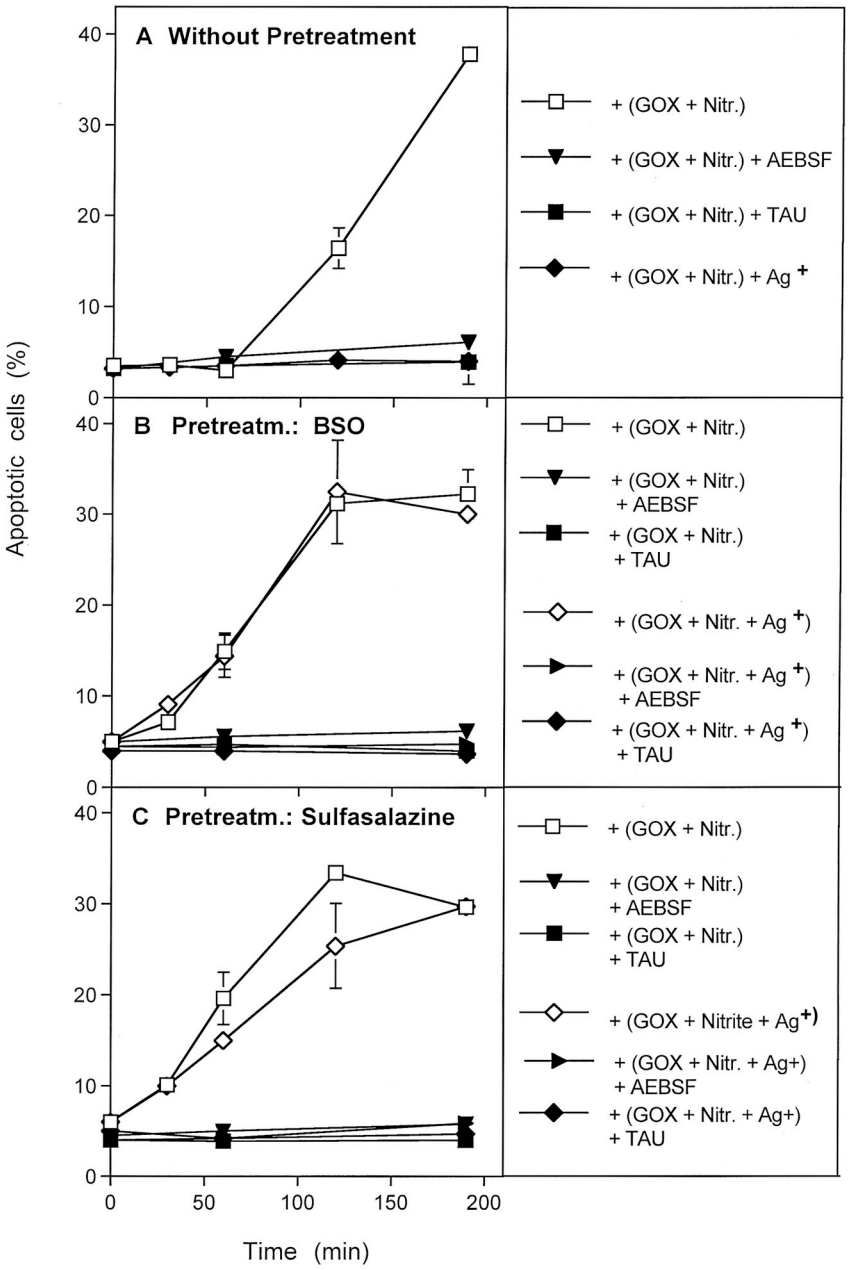


Fig. 13. BSO- and sulfasalazine-pretreatment does not change the quality of intercellular signaling after application of GOX/nitrite.
The figure shows an extension of the experiment described in Fig. 12, where assays containing GOX and nitrite (without or with Ag^+) from untreated cultures or cultures that had been pretreated with either BSO or sulfasalazine received in addition the NOX1 inhibitor AEBSF or the HOCl scavenger taurine.
The results show that under all conditions that allowed HOCl signaling in cells without pretreatment, HOCl signaling was also demonstrated in BSO- or sulfasalazine-pretreated cells. This indicates that the modulation of the glutathione level was without effect on the quality of intercellular signaling, though this signaling occurred faster and was independent of aquaporins.
Statistical analysis: AEBSF and taurine cause highly significant ($p < 0.001$) inhibition of apoptosis induction in GOX/nitrite-treated cells, irrespective of previous pretreatment with BSO or sulfasalazine, or the absence or presence of Ag^+.

primarily less reactive molecular species. The secret of their biological effectiveness is their potential to generate singlet oxygen in close vicinity to living cells and to induce a burst of secondary singlet oxygen, if the targeted cells are tumor cells with their specific redox elements on their surface.

The role of primary singlet oxygen generated by CAP or PAM-derived H_2O_2 and nitrite is to trigger the tumor cells to contribute to their own cell death through the subsequent generation of secondary singlet oxygen, catalase inactivation, and establishment of intercellular apoptosis inducing signaling. As the generation of secondary singlet oxygen, as well as intercellular apoptosis-inducing signaling requires tumor cell-specific redox elements like catalase and NOX1, selectivity of apoptosis induction in tumor cells by CAP and PAM is warranted, as long as the overall concentration of H_2O_2 in CAP or PAM is sufficiently low to not affect nonmalignant cells. The efficiency of this process is determined by its sustained nature. According to our model, the long-lived species H_2O_2 and nitrite from CAP and PAM contribute a minute, but highly effective triggering part to the overall process, whereas tumor cell-derived ROS/RNS drive the major part of ROS/RNS-dependent processes during their own selective cell death. This is clearly different from the concepts established by Yan et al., Van Paal et al. and Van Boxem et al. [81–84], in which CAP or PAM-derived H_2O_2 has been suggested to directly cause cell death in tumor cells. These models also neglected that membrane-associated catalase of tumor cells represents a strong barrier for exogenous H_2O_2, whereas nonmalignant cells are unprotected [43].

Our experimental analysis allowed to draw a model that comprises three major steps for apoptosis induction in tumor cells by long-lived species derived from CAP and contained in PAM (Fig. 16 A). These are singlet oxygen-dependent inactivation of catalase (step #1), aquaporin-mediated influx of H_2O_2 into the cells (step #2) and intercellular apoptosis-inducing signaling (step #3).

Step #1 was initiated by the interaction between CAP or PAM-derived H_2O_2 and nitrite, mediated by singlet oxygen, and was completed within 30 min. It finally caused substantial inactivation of membrane-associated catalase. Step 1 was composed of two distinct, but tightly

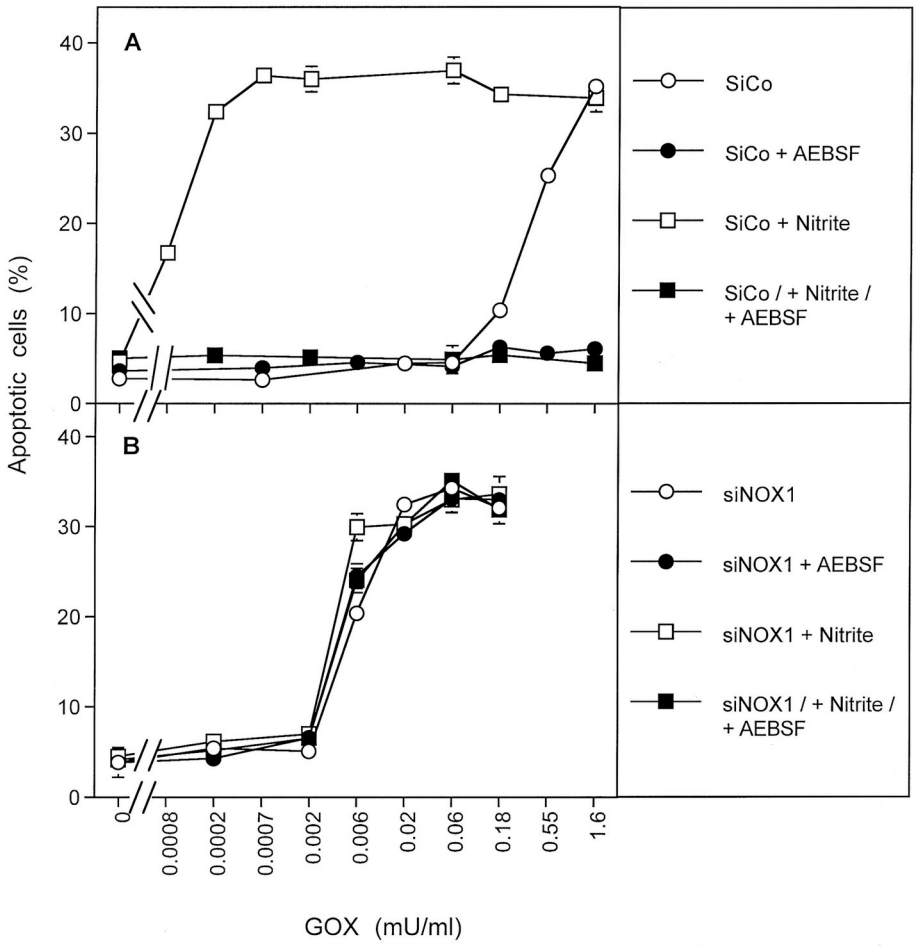


Fig. 14. Determination of the windows for selective and nonselective action of GOX/nitrite towards tumor cells. SiRNA-based analysis.
MKN-45 cells were transfected with irrelevant control siRNA (siCo) or siRNA directed towards NOX1 (siNOX1). After 24 h, the cells were washed and resuspended in fresh medium. Parallel controls ensured that this treatment had caused more than 90% knockdown of NOX1 and had also caused subsequent downmodulation of catalase, as catalase expression is controlled by active NOX1.
SiCo and siNOX1 cells were treated with the indicated concentrations of GOX, in the absence or presence of the indicated additions (nitrite 1 mM; AEBSF 100 μM). The percentages of apoptotic cells were determined after 3 h.
A. Control cells (SiCo).The results confirm the synergistic effect between GOX and nitrite in a wide concentration range, up to 0.18 mU/ml GOX, which was followed by apoptosis induction even in the absence of nitrite at higher concentrations of GOX. Both processes depended on active NOX1, as they were completely inhibited by AEBSF.
B. SiNOX cells (siNOX1) lack the tumor cell phenotype that is characterized by expression of NOX1 and membrane-associated catalase. These cells are much more sensitive towards H_2O_2-mediated apoptosis induction than siCO cells. There was no indication for a synergistic effect between H_2O_2 and nitrite on these cells, and apoptosis induction under all conditions applied was independent of NOX1-derived superoxide anions.
These data allow to define a window of selective apoptosis induction in tumor cells without damaging cells with the nonmalignant phenotype.
Statistical analysis: A: Apoptosis induction in the presence of GOX or GOX/nitrite is highly significant ($p < 0.001$). The differences in the concentration dependency of these two processes, as well as their inhibition by AEBSF are highly significant ($p < 0.001$). B: Apoptosis induction by all four combinations of compounds are highly significant ($p < 0.001$), but there are no significant differences in their concentration dependencies. However, the concentration dependency of the group of assays shown under B are highly significant ($p < 0.001$) from the analogous assays under A.

interacting substeps. Substep #1 required the generation of low concentrations of “primary singlet oxygen” through the interaction between nitrite and H_2O_2, leading to the formation of peroxynitrite. Peroxynitrite and residual CAP/PAM-derived H_2O_2 then allow for the formation of primary singlet oxygen [47,85]. After local inactivation of few catalase molecules on the surface of the tumor cells by primary singlet oxygen, peroxynitrite and H_2O_2 generated by the tumor cells than allow for the sustained generation of secondary singlet oxygen (substep #2). Secondary singlet oxygen seems to drive autoamplification of singlet oxygen generation as well as further inactivation of catalase, as determined by inhibitor studies and direct evaluation of catalase inactivation through an exogenous peroxynitrite challenge.

2) The subsequent aquaporine-mediated step (step #2) was nearly completed 1 h after addition of GOX and nitrite. It seemed to comprise intracellular sensitization of the cells for apoptosis induction by ROS signaling, as inhibition of this step by Ag^+ prevented apoptosis induction despite inactivated catalase and ongoing HOCl signaling. Sensitization can be explained by glutathione depletion that renders the repair of lipid peroxidation by glutathione peroxidase-4/glutathione ineffective [77], as experimental glutathione depletion through BSO and sulfasalazine before application of H_2O_2/nitrite rendered apoptosis induction through ROS signaling independent of the aquaporin function.

3) A subsequent longer-lasting step (step 3) was no longer dependent on direct singlet oxygen action. It was due to intercellular apoptosis-inducing signaling through the HOCl signaling pathway, followed by the mitochondrial pathway of apoptosis and driven by caspases-9 and -3.

Apoptosis-inducing HOCl signaling after CAP/PAM treatment of cells strictly requires the preceding completion of steps #1 and #2. It is therefore double-controlled by two interconnected steps. Catalase inactivation is the essential key for the onset for HOCl signaling and for H_2O_2 influx through aquaporins.

The lag phase of about 1 h after addition of H_2O_2/nitrite to tumor cells can be explained by the requirement for singlet oxygen generation, singlet oxygen-mediated catalase inactivation and the aquaporine-mediated sensitization step through influx of H_2O_2, before HOCl signaling is established and is effectively inducing apoptosis (Fig. 16).

Experimental dissection of this biological system allowed to study step #1 - #3 independent of each other, and to determine the molecular species that were involved in each individual step (Fig. 16 B). The complexity of these steps requires individual discussion of the underlying biochemistry of each step. Analysis and interpretation are especially challenging, as several molecular species are involved in several steps, and few are acting specifically at only one defined step. This interconnection may be the basis for rather tight and coordinated control of these steps.

4.1. Biochemical analysis of step #1

The proposed concept of generation of primary and secondary singlet oxygen and its action on membrane-associated catalase is based on several established findings (Fig. 17 A):

1) As nitrite **and** H_2O_2 are both required to trigger apoptosis induction

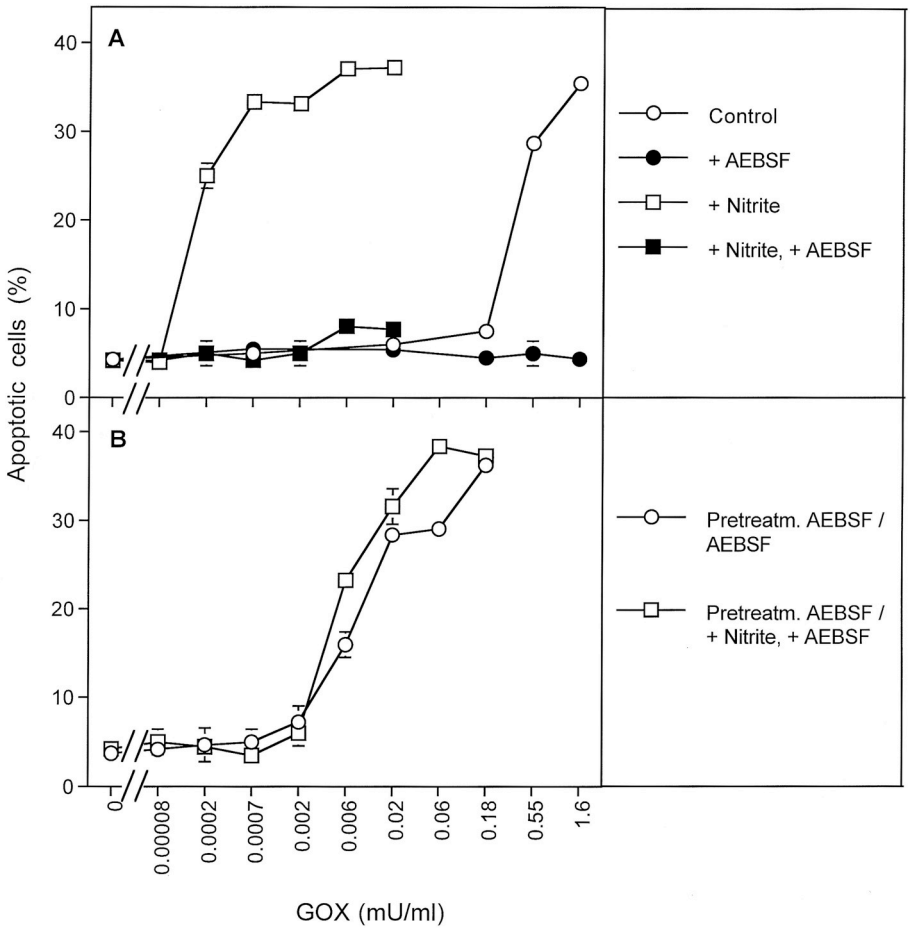


Fig. 15. Determination of the windows for selective and nonselective action of GOX/nitrite towards tumor cells. Inhibitor-based analysis.
The experiment was performed in a mode analogous to the experiment described in Fig. 14, with the exception that tumor cells were not pretreated with siRNA, but cells described under B had been pretreated in the presence of 100 μM AEBSF for 14 h. Inhibition of NOX1 for 14 h has a profound negative effect on the expression of catalase [43]. Therefore, the cells pretreated with AEBSF and further cultivated in the presence of AEBSF had a NOX1 negative/catalase negative phenotype. Treatment of these cells with GOX, in the absence or presence of nitrite showed that they were more sensitive to apoptosis induction by H_2O_2 and were not able to respond to the a synergistic effect between H_2O_2 and nitrite that is shown for control cells.
Statistical analysis: A: Apoptosis induction in the control assays and in the presence of additional nitrite, as well as their inhibition by AEBSF are highly significant ($p < 0.001$). The differences in the concentration dependencies are highly significant ($p < 0.001$). B: Apoptosis induction in both groups is highly significant ($p < 0.001$). There are no significant differences with respect to concentration dependencies between the two groups, but highly significant differences ($p < 0.001$) when the groups shown in B are compared to the respective groups under A.

in tumor cells, their reaction product peroxynitrite seems to play a role according to Lukes et al. [33]

$$NO_2^- + H_2O_2 \rightarrow ONOO^- + H_2O \quad (1)$$

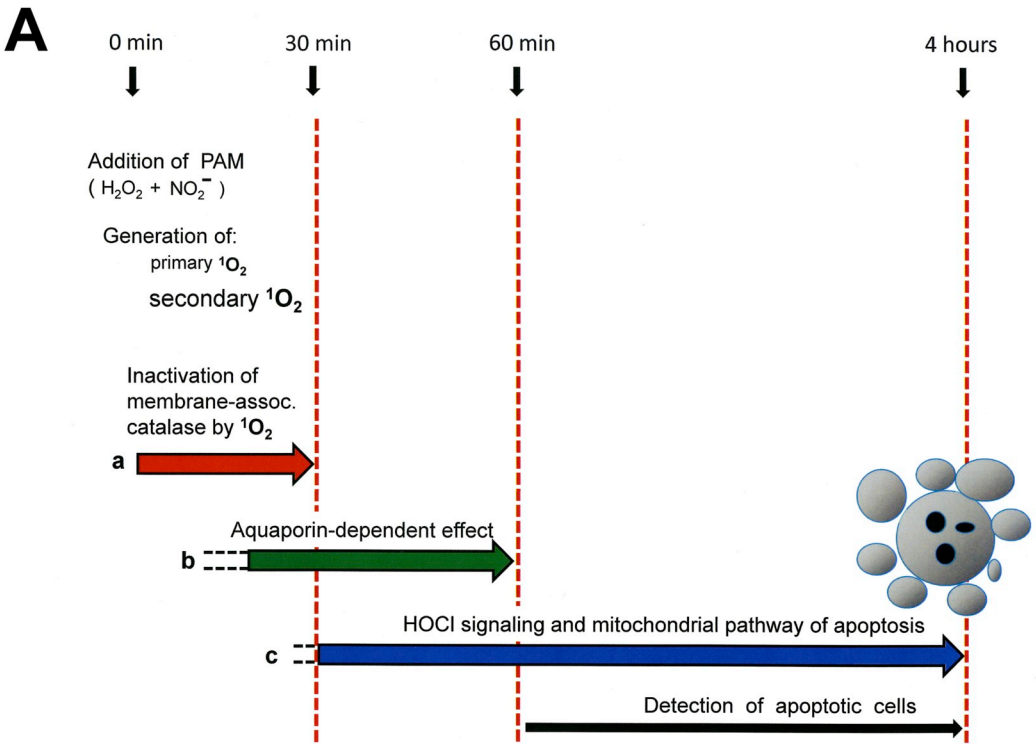
2) As membrane-associated catalase of tumor cells is efficiently decomposing peroxynitrite [42], a direct apoptosis-inducing effect of peroxynitrite derived from nitrite/H_2O_2 interaction can be excluded. Furthermore, peroxynitrite formation is rather slow and the steady state concentration of peroxynitrite has been reported to be low as well [33].
3) Due to the slow reaction of H_2O_2 with nitrite, peroxynitrite as well as H_2O_2 can be predicted to be coexisting in the assays.Therefore, the interaction of these two species should allow the formation of primary singlet oxygen, according to the findings by Di Masico et al. [85]. These authors were the first to demonstrate that the interaction between H_2O_2 and peroxynitrite caused the generation of singlet oxygen. They proposed the Russel reaction as underlying mechanism for singlet oxygen generation (Fig. 17 A).
4) Primary singlet oxygen may then cause local inactivation of few molecules of membrane-associated catalase through reaction with a histidine residue in their active center [86,87].
5) As deduced from model experiments with the singlet oxygen generator photofrin, local inactivation of catalase on tumor cells alone is not sufficient to reactivate intercellular apoptosis-inducing ROS/RNS signaling [57]. Rather, massive generation of secondary singlet oxygen by the tumor cells, followed by substantial inactivation of catalase seems to be required. In line with this conclusion, inhibition of NOX1 with AEBSF and NOS with L-NAME completely prevented catalase inactivation by nitrite/H_2O_2 interaction. ***This points to the dominating role of secondary singlet generation in this scenario.***
6) Local inactivation of catalase generates a site at which tumor cell-derived H_2O_2 and peroxynitrite are not decomposed. This allows for the generation of secondary singlet oxygen in a sustained mode, as the required precursors for singlet oxygen generation, i. e. H_2O_2 and peroxynitrite, are constantly produced due to the activity of NOX1 and NOS.
7) As a result, locally generated secondary singlet oxygen may inactivate neighbouring catalase molecules on the surface of the tumor cells and then establish autoamplificatory $^1O_2$ generation. This process is further enhanced by singlet oxygen-dependent activation of the FAS receptor and the resultant stimulatory effects on NOX1 and NOS [50,57].

The scheme shown in Fig. 17 A is in line with the inhibition of catalase inactivation by nitrite/H_2O_2 in the presence of histidine, FeTPPS, EUK-134, AEBSF, L-NAME and caspase-8 inhibitor.

However, this scheme does not explain the strong inhibitory effect of the hydroxyl radical scavenger mannitol on catalase inactivation that has been found in our experiments.

Furthermore, though the generation of singlet oxygen after interaction between H_2O_2 and peroxynitrite as determined by DiMascio et al. [85] (Fig. 17 A) remains undisputed, the Russel reaction suggested by these authors as underlying mechanism is directly contradicted by the data obtained by Alvarez et al. [88]. These authors showed that two molecules of peroxynitrite are required to obtain one molecule of singlet oxygen, whereas the Russel mechanism would require only one molecule of peroxynitrite per one molecule of singlet oxygen.

A role for a dominant function of hydroxyl radicals during the process of inactivation of catalase by nitrite/H_2O_2 treatment can be suggested, when the reaction between hydroxyl radicals (derived from



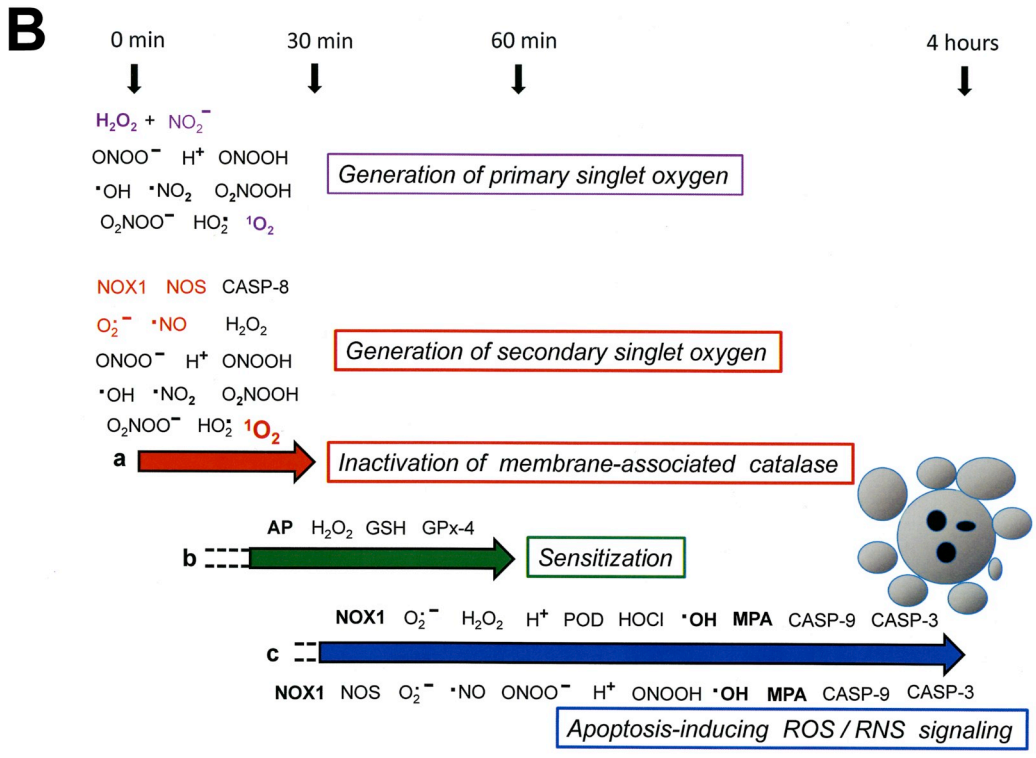


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Fig. 16. Distinct steps and substeps during apoptosis induction in tumor cells by the synergistic effect between H_2O_2 and nitrite.
A. Basic steps and substeps. a) Addition of plasma-activated medium, i. e. the long-lived species derived from CAP action to tumor cells is mimicked in this study by the essential model compounds H_2O_2 and nitrite. Within less than 30 min, the interaction of H_2O_2 and nitrite leads to the formation of primary singlet oxygen, subsequent generation of secondary singlet oxygen and finally substantial inactivation of protective catalase. b) at the site of inactivated catalase, the influx of H_2O_2 into the cells is possible. This causes intracellular depletion of GSH and thus the effect of glutathione peroxidase-4/GSH on lipid peroxidation is abrogated. This step is completed after about 60 min and sensitizes the cells for apoptosis induction by intercellular apoptosis-inducing ROS signaling. c) Intercellular apoptosis-inducing signaling through the HOCl signaling pathway requires preceding inactivation of catalase and aquaporine-mediated GSH depletion. It leads to lipid peroxidation and the induction of the mitochondrial pathway of apoptosis.
B. Molecular species involved in the essential steps. Generation of primary singlet oxygen is based on the generation of peroxynitrite through the interaction between H_2O_2 and nitrite, followed by singlet oxygen generation through H_2O_2/peroxynitrite interaction as shown in Fig. 17 B, involving the listed species as intermediates.Generation of secondary singlet oxygen by the tumor cells is based on NOX1 and NOS that lead to the generation of cell-derived H_2O_2 and peroxynitrite. Their interaction leads to the formation of singlet oxygen as shown in Fig. 17 B.
Inactivation of catalase is essentially mediated by secondary singlet oxygen. Sensitization of tumor cells is achieved through influx of H_2O_2 through aquaporins.
Intercellular apoptosis-inducing signaling as studied here was driven by NOX1, mediated by the compounds of the HOCl signaling pathway and finalized by the mitochondrial pathway of apoptosis (MPA) using caspase-9 and -3. Supplementation with NO allowed for the establishment of the NO/peroxynitrite signaling pathway.

decomposition of peroxynitrous acid) with H_2O_2 is considered. (Fig. 17 B). The resultant hydroperoxyl radicals may then interact with NO_2, the second decomposition product of peroxynitrous acid, and generate peroxynitric acid (O_2NOOH) [89]. This opens the path for singlet oxygen generation from peroxynitrate [90].

Based on the inhibitor data obtained in this study, on established chemical biology of the molecular species involved and on related singlet oxygen-dependent processes [47,57,74], the following sequence of primary and secondary singlet oxygen generation initiated by H_2O_2/nitrite interaction with tumor cells is therefore proposed:

A. Generation of primary singlet oxygen :

$$NO_2^- + H_2O_2 \rightarrow ONOO^- + H_2O \quad (2)$$

$$ONOO^- + H^+ \rightarrow ONOOH \quad (3)$$

$$ONOOH \rightarrow {}^{\bullet}NO_2 + {}^{\bullet}OH \quad (4)$$

$$^{\bullet}OH + H_2O_2 \rightarrow H_2O + HO_2^{\bullet} \quad (5)$$

$$^{\bullet}NO_2 + HO_2^{\bullet} \rightarrow O_2NOOH \quad (6)$$

$$O_2NOOH \rightarrow O_2NOO^- \quad (7)$$

$$O_2NOO^- \rightarrow {}^1O_2 + NO_2^- \quad (8)$$

$$O_2NOO^- \rightarrow O_2^{\bullet -} + {}^{\bullet}NO_2 \quad (9)$$

Detailed information about equation (2) can be found in reference [33], about (4) in [91] and [92], about (5) in [93], about (6) in [89], about (8) and (9) in [89] and [90].

This sequence of reactions is supported by the requirement for exogenous H_2O_2 and nitrite, by the inhibitory effects of EUK-134 (catalase mimetic), FeTPPS (peroxynitrite decomposition catalyst), mannitol (hydroxyl radical scavenger) and histidine (singlet oxygen scavenger) (Fig. 17 B). Furthermore, Girard et al. [94] have confirmed that peroxynitrite can be detected after CAP treatment of buffer at neutral pH. However, peroxynitrite cannot cause apoptosis induction of tumor cells directly, as it is efficiently decomposed by membrane-associated catalase [42,43], but its role for the generation of singlet oxygen is essential. The role of peroxynitric acid/peroxynitrate was not directly established by inhibitor experiments in this study, as no discriminative inhibitors for these two compounds are available. However, Kitano et al.,Ikawa et al. and Liu et al. [95–97] have demonstrated the presence and functionality of peroxynitric acid (O_2NOOH) and peroxynitrate (O_2NOO^-) in PAM. This is in line with our concept and therefore supports the suggested reaction mechanism. Based on the short-lived nature of peroxynitrate/peroxynitric acid, it seems rather unlikely that peroxynitrate/peroxynitric acid that is detectable in PAM was generated during the interaction between CAP and medium. It seems more likely that the occurrence of peroxynitrate/peroxynitric acid in PAM is due to the interaction between the long-lived species nitrite and H_2O_2, as delineated in equations (2)–(8) above.

As protonation of peroxynitrite is favoured by proton pumps in close vicinity of the cell membrane, the alternative reaction between peroxynitrite and CO_2 [98–100] is outcompeted there and the formation of peroxynitrous acid is favoured. Therefore, the crucial reactions of generation and subsequent decomposition of peroxynitrous acid are exactly located at the right site, i. e. at the membrane, where the generation of primary singlet oxygen is required for initial inactivation of membrane-associated catalase. $^1O_2$ generated at this site, has a chance to actually reach its target. It can be predicted that the generation of singlet oxygen is restricted to a zone above the cell membrane where peroxynitrite and H_2O_2 are out of reach of the decomposing activity of catalase that is bound to the membrane, but sufficiently close to the proton pumps that are required for the generation of peroxynitrous acid.

After local inactivation of membrane-associated catalase, secondary singlet oxygen is generated at the site of inactivated catalase through the following reactions [101]:

$$2\ O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + O_2 \quad (10)$$

This is achieved through NOX1 activity and subsequent spontaneous or SOD-driven dismutation. Then NOX-derived superoxide anions and NOS-derived NO generate peroxynitrite [61,91,102–104]:

$$O_2^{\bullet -} + {}^{\bullet}NO \rightarrow ONOO^- \quad (11)$$

Tumor cell-generated H_2O_2 and $ONOO^-$ then interact according to equations (2)–(8) as shown above, following the same sequence of biochemical events as delineated for primary singlet oxygen generation.

Thus, there is a strong overlap between the mechanisms of generation of primary and secondary singlet oxygen. The discriminating difference is localized at the onset of the reactions: primary singlet oxygen generation starts with H_2O_2 derived from CAP and peroxynitrite generated through the interaction between CAP-derived H_2O_2 and nitrite, whereas secondary singlet oxygen generation starts directly with H_2O_2 and peroxynitrite supplied by NOX1 and NOS of the tumor cells. Whereas primary singlet oxygen generation seems to be a rare and limiting process, the generation of secondary singlet oxygen is sustained and continuously driven by NOX and NOS, as soon as local inactivation of catalase has been achieved by primary singlet oxygen.

The activity of these two enzymes is further enhanced by singlet oxygen-mediated FAS receptor activation [[105–108], reviewed in Ref. [50]].

The partial congruence as well as the overlap between the mechanisms of primary and secondary singlet oxygen generation were confirmed in a further analysis [Bauer, adjacent manuscript]. This extended study dissected step #1 into the initial rare primary singlet oxygen generation and subsequent massive secondary singlet oxygen generation, thereby demonstrating the dynamics of autoamplificatory bystander signaling in more detail.

The mechanisms of primary and secondary singlet oxygen

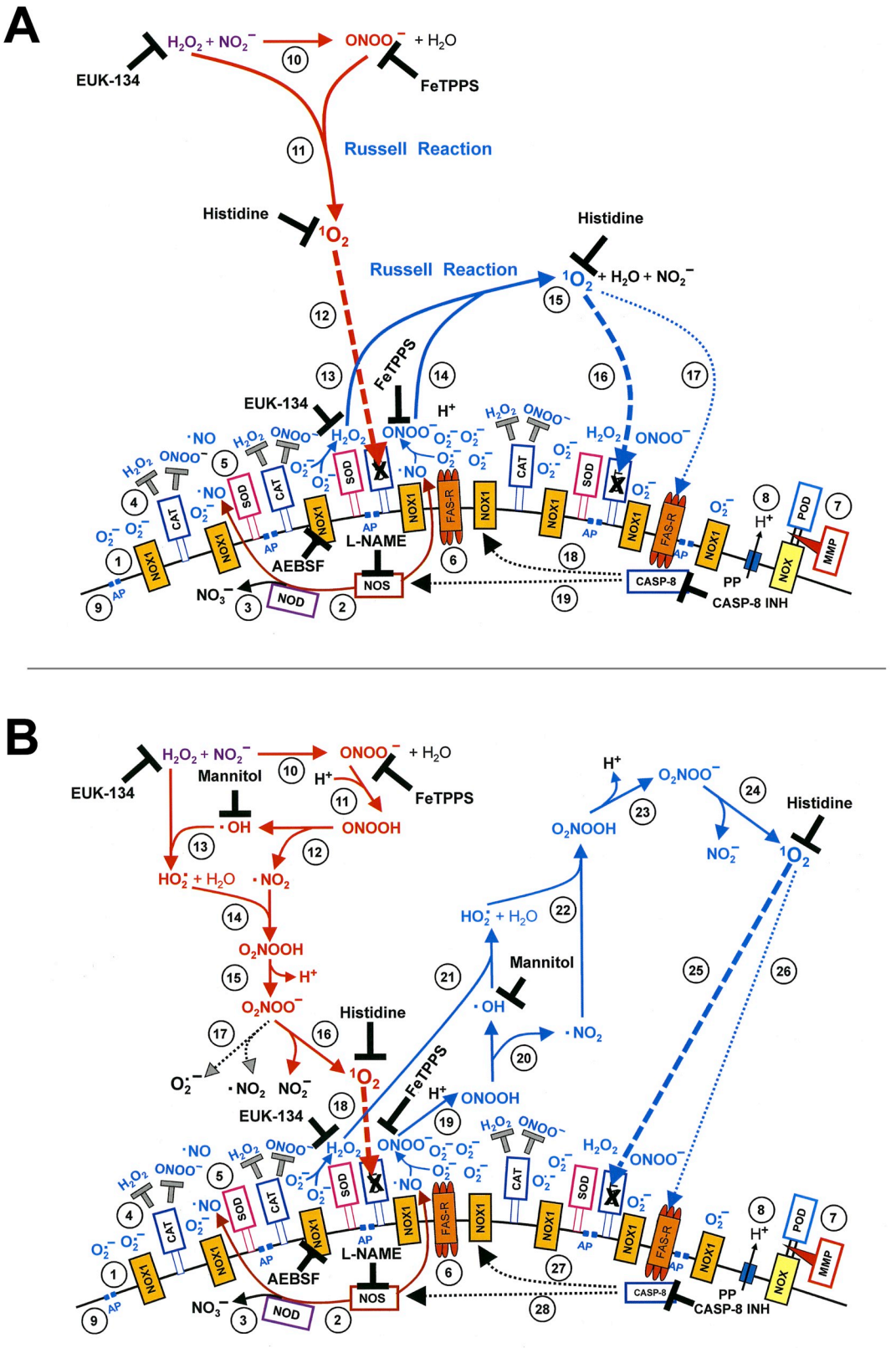

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Fig. 17. Alternative schemes for the biochemical mechanisms for the generation of primary and secondary singlet oxygen.
A. NADPH oxidase 1 (NOX1) is expressed in the membrane of malignant cells and generates extracellular superoxide anions (#1). NO synthase (NOS) (#2) generates NO which can be either oxidised by NO dioxygenase (NOD) (#3) or pass through the cell membrane. Membrane-associated catalase (#4) protects tumor cells towards intercellular ROS/RNS-mediated signaling through decomposition of H_2O_2 and peroxynitrite, as well as oxidation of NO (not shown in the figure). Comodulatory SOD (#5) is required to prevent superoxide anion-mediated inhibition of catalase. Further important elements in the membrane are the FAS receptor (#6), Dual oxidase (DUOX) (#7), from which a peroxidase domain is split through matrix metalloprotease, proton pumps (#8) and aquaporins (#9).
H_2O_2 and nitrite, two long-lived species from plasma-activated medium, interact and generate peroxynitrite (#10) [33]. The interaction between peroxynitrite and residual H_2O_2 allows for the generation of singlet oxygen (#11) through the Russell reaction, according to DiMasico et al. [85]. This primary singlet oxygen causes local inactivation of catalase (#12). As a consequence, generation of secondary singlet oxygen by the tumor cells is established at the site of inactivated catalase, where H_2O_2 (#13) and peroxynitrite (#14) generate secondary singlet oxygen ($^1O_2$) (#15). Singlet oxygen may then either inactivate another molecule of catalase (#16) or activate the FAS receptor (#17). This causes activation of NOX1 and NOS activities (#18, #19), which causes further enhancement of singlet oxygen generation. Fig. 17 A indicates the site of action of specific inhibitors/scavengers that interfere with the outlined pathways. It is obvious, that the experimentally determined role of hydroxyl radicals (scavenging by mannitol) is not explained by this scheme.
B. Completed picture of primary and secondary singlet oxygen generation.Steps # 1 – #10 in Fig. 17 B are identical to those shown in Fig. 17 A. Rather than direct interaction between H_2O_2 and peroxynitrite, the sequence defined by steps # 11 - #16 is suggested. It is in line with all inhibitory data established and with the findings in the literature. It is based on generation of peroxynitrous acid (#11), generation of hydroxl radicals through decomposition of peroxynitrous acid (#12), and on hydroperoxyl radicals (#13) that are derived from hydroxyl radical/H_2O_2 interaction. The interaction between hydroperoxyl radicals and NO_2 leads to the generation of peroxynitric acid (O_2NOOH) (#14). Deprotonation of peroxynitric acid establishes the formation of peroxynitrate (#15) and the generation of singlet oxygen (#16) that causes local inactivation of catalase (#18). The generation of secondary singlet oxygen starts with free H_2O_2 (#18) and peroxynitrite (#19), followed by reactions #20 - #24, in analogy to the reactions described for primary singlet oxygen.The scheme presented in Fig. 17 B is in perfect agreement with all of our experimental findings and the literature.

generation as described in Fig. 17 B seems to be more conclusive than alternative mechanistic suggestions, mainly as these are not in line with the inhibitor data obtained in this study.

The theoretical schemes for primary and secondary singlet oxygen formation shown in Fig. 18 A are in principal agreement with most of our findings. However, they do not include any role of peroxynitrate/peroxynitric acid, but rather explain the final step of singlet oxygen generation through the interaction between hydroperoxyl radicals:

$$2\ HO_2^{\bullet} \rightarrow H_2O_2 + {}^1O_2 \quad (12)$$

A large number of publications is in favour of singlet oxygen generation through hydroperoxyl interaction [101,109–118]. Therefore, our initial model on the potential CAP mechanism [76] also contained this detail of singlet oxygen generation through hydroperoxyl radical interaction. None of the publications that favour hydroperoxide interaction during singlet oxygen generation has been retracted to my knowledge. Nevertheless, singlet oxygen generation through the reaction between two hydroperoxyl radicals or between superoxide anions and hydroperoxyl radicals has often been questioned and strongly disputed [119–122]. In addition, the scheme shown in Fig. 18 A does not take into account the actual finding of peroxynitrate and peroxynitric acid by Kitano et al. Ikawa et al. and Liu et al. [95–97]. Our view on these aspects gets further supported by the finding on the central role of PAM-contained peroxynitric acid for its antibacterial effects [95–97] and the finding on the strong potential of PAM-derived singlet oxygen for the killing of bacteria [123]. This connection allows to speculate on an analogous mechanistic setup of antibacterial effects of PAM and the selective antitumor effects as studied here. Therefore, our present view favours the reaction schemes shown in Fig. 17 B, rather than the schemes in Fig. 18 A.

Finally, the modification of the reaction scheme as shown in Fig. 18 B, in which peroxynitric acid is suggested to be directly generated through the reaction between peroxynitrite and H_2O_2 [95] is not in line with our inhibitor data, as it does not include the experimentally determined dominant role of hydroxyl radicals.

- The most important and novel conclusion from our data related to step 1 is the concept that it is sufficient for PAM- and CAP-derived species to act as a trigger that interacts with the switchboard on the tumor cells. This then induces an tumor cell-driven, strong auto-amplificatory mechanism for tumor cell destruction. Therefore, only low concentrations of triggering compounds need to be present and generated in PAM and CAP. These do not harm non-malignant cells, as these cells do not possess the necessary switchboard, whereas in tumor cells a massive self-sustaining chain of effects, driven by their malignant features, establishes a selective antitumor effect. Work in progress shows that the concept established by using a H_2O_2 source in combination with nitrite is congruent with the mechanism of apoptosis induction in tumor cells by a plasma source [Bauer et al., manuscript submitted].

4.2. Biochemical analysis of step 2

As soon as step 1 is completed, the subsequent steps 2 and 3 are independent of the presence of CAP- or PAM-derived molecular species, as they are completely driven by tumor cell-specific enzymes. This is explicitely proven by the fact that intercellular apoptosis inducing signaling (step 3) is occurring after removal of PAM and CAP-related species after completion of step 1, as shown in Fig. 11.

As intercellular apoptosis inducing signaling is inhibited by the aquaporine inhibitor Ag^+, step 2 seems to occur before the onset of apoptosis induction, but is not completed at the point of optimal catalase inactivation by singlet oxygen. It seems to consist of influx of cell-derived extracellular H_2O_2 through aquaporins, leading to a depletion of intracellular glutathione. This conclusion is based on the finding that the aquaporin-mediated step can be mimicked experimentally through depletion of glutathione before the experiment. This can be predicted to prevent glutathione peroxidase-4 dependent correction of lipid peroxidation [77]. In this way the interference of glutathione peroxidase-4/glutathione with hydroxyl radical-mediated apoptosis induction through lipid peroxidation is abrogated and the cells are sensitized for the biochemical effects of intercellular apoptosis-inducing signaling. Importantly, the aquaporin-dependent step is a central step, but seems to occur only after inactivation membrane-associated catalase, as catalase is preventing the influx through aquaporins. Therefore, our findings do not favour the concept of primary, determining function of aquaporins for determination of selectivity of CAP and PAM action, according to the model presented by Yan et al. [81,82]. We strongly agree with Yan et al., that the role of aquaporins is dominant and central. However, according to our findings, an important aquaporin-dependent step is located between step 1 and step 3. Our suggestion for the dominant and therefore important function of aquaporins is explicitely shown in Fig. 11 A and B, as intercellular HOCl as well as NO/peroxynitrite signaling after catalase inactivation were completely prevented when aquaporine function had been inhibited. Importantly, the biological role of aquaporins was only significant in tumor cells with preceding inactivation of membrane-associated catalase by CAP or PAM.

Step #3 in the experimental system presented here was classical

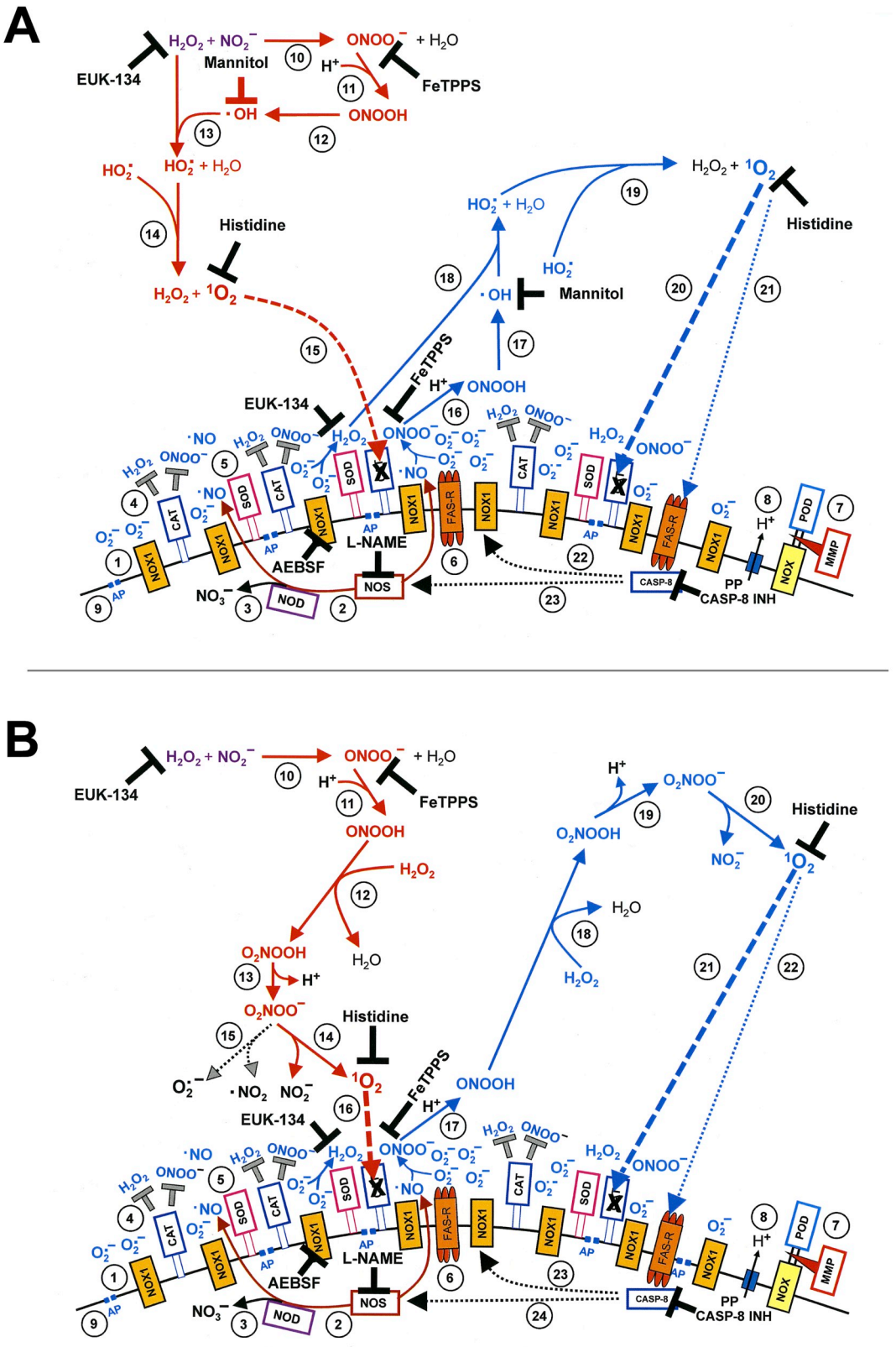
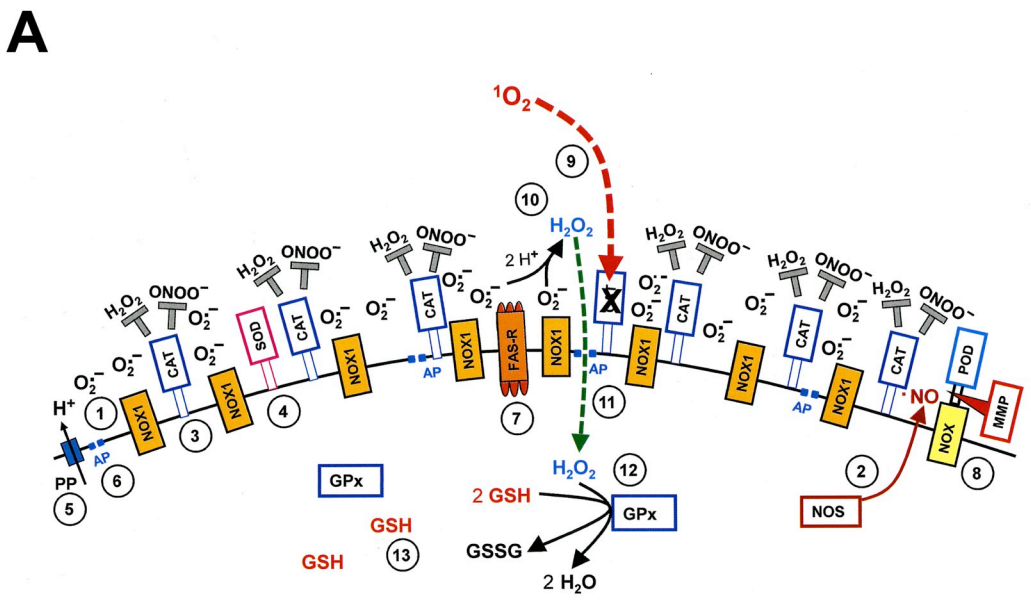


Fig. 18. Alternative mechanisms for the generation of primary and secondary singlet oxygen.
A. This scheme differs from the preferred scheme shown in Fig. 17 B with regard to the reaction of hydroperoxyl radicals with each other (# 14), leading to the generation of singlet oxygen. In Fig. 17B, hydroperoxyl radicals are proposed to react with NO_2, instead. The scheme shown in Fig. 18 A is in principle agreement with all of our inhibitor data, but is (partially) disputed by the literature (please find details under Discussion).
B. Generation of peroxynitric acid through interaction of peroxnitrous acid with H_2O_2, as suggested here, leads to a reaction scheme that does not include any role of hydroxyl radicals. It is therefore not in agreement with our inhibitor data. Therefore, even if this reaction has a chance to occur under the experimental conditions, its contribution to the overall process seems to be neglectable.



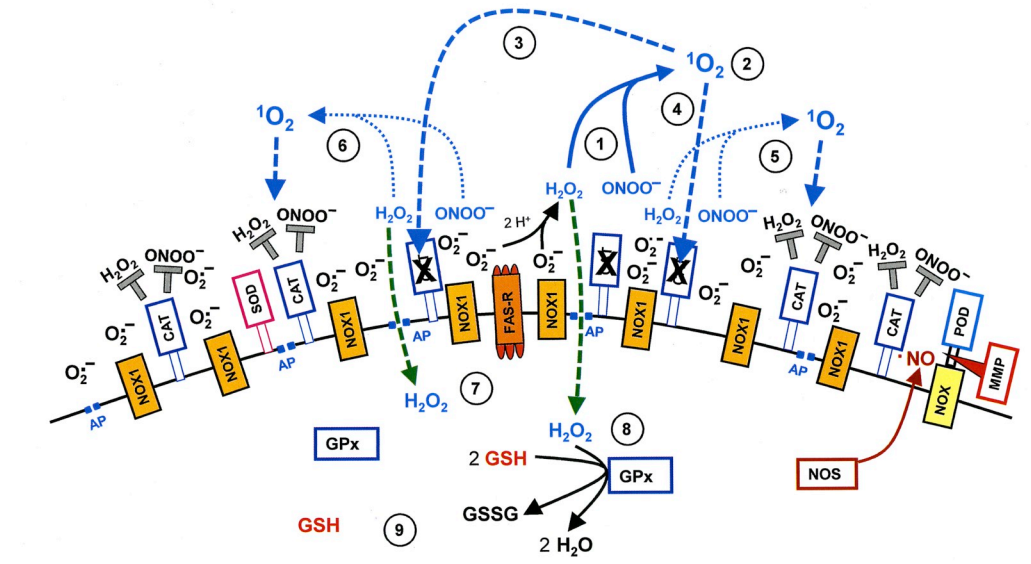


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Fig. 19. Apoptosis induction in tumor cells by primary singlet oxygen generated through the interaction of the longlived species H_2O_2 and nitrite from CAP/PAM.
A. The membrane of tumor cells is characterized by expression of NOX1 that generates extracellular superoxide anions (#1). NOS activity generates NO that passes the cell membrane (#2). Membrane-associated catalase protects towards HOCl and NO/peroxynitrite signaling through decomposition of H_2O_2 and nitrite (#3). SOD ensures catalase activity through partial removal of inhibitory superoxide anions (#4). Other essential elements are proton pumps (#5), aquaporins (#6), the FAS receptor (#7) and Dual oxidase (DUOX), consisting of a NOX and peroxidase domain (#8). Primary singlet oxygen ($^1O_2$) (#9) generated through the interaction between H_2O_2 and nitrite (as outlined in Fig. 17 B) causes local inactivation of catalase.
At the site of inactivated catalase, H_2O_2 generated through dismutation of superoxide anions (#10) has a chance to pass into the cell through aquaporins (#11). I twill be subsequently be decomposed by glutathione peroxidase-4/glutathione (# 12) and thus lower the intracellular level of GSH.
B. At the site of inactivated catalase, free cell-derived H_2O_2 and peroxynitrite have a chance to interact (#1) and generate secondary singlet oxygen (#2) according to the reactions defined inFig. 17 B. *Please note that for simplicity of the graph, this complex interaction is summarized here as two simple lines*. Secondary singlet oxygen causes inactivation of further catalase molecules (#3, #4), leading to amplification of secondary singlet oxygen generation (#5, 6). As a consequence, intrusion of H_2O_2 into the cells will increase (#7) and lead to further decrease in the concentration of GSH (#8, #9).

HOCl signaling, as described in several other systems of control of malignant cells [44,60,61] (Fig. 20 A). It is based on dismutation of superoxide anions to H_2O_2, use of H_2O_2 of the peroxidase domain of DUOX for the generation of HOCl [44] and HOCl/superoxide anion interaction leading to the generation of hydroxyl radicals [124,125]. These induce lipid peroxidation and thus trigger the mitochondrial pathway of apoptosis (Fig. 20 B).

Expression of HOCl signaling requires optimal inactivation of tumor cell protective catalase. At less sufficient catalase inactivation, or after experimental supply with an NO donor, NO/peroxynitrite signaling is conceivable [45].

This picture includes the data derived from this study and from previous work of my group. It fully includes the important findings by Keidar's group on the dominant role of aquaporins for CAP and PAM effects [82,83]. However, the time of action of aquaporins has been determined in this study to be located after inactivation of membrane-associated catalase. Otherwise, catalase would prevent H_2O_2 influx through aquaporins.

Bekeschus et al. [126]. detected no extracellular singlet oxygen in close vicinity to the cell membrane during the initial steps of CAP action, but measured mitochondrial singlet oxygen late after plasma treatment. The nondetectibility of singlet oxygen that, according to our functional measurements, seems to be relevant initially, might be explained by the low overall concentration of singlet oxygen and its generation in close vicinity to the belt of membrane-associated catalase. Catalase, with its histidine-containing active center might act as a competing scavenging system that counteracted the detection. The study by Bekeschus et al. did also not include testing of functional relevance of singlet oxygen. Singlet oxygen generation during the late phase of CAP action, i. e. during the execution of the mitochondrial pathway is not unexpected, as the release of cytochrome c from the mitochondria during the mitochondrial pathway of apoptosis causes mitochondrial dysfunction and massive generation of superoxide anions and H_2O_2. In light of the recently determined potential of cytochrome c to use H_2O_2 as substrate for HOCl synthesis [Bauer, unpublished result), singlet oxygen generation through the interaction between H_2O_2 and HOCl [127] seems feasible. However, as late addition of the singlet oxygen scavenger histidine had no inhibitory effect on apoptosis induction in our study, it seems more likely that this late singlet oxygen generation reflects a consequence rather than a cause of apoptosis induction.

This study was focused on the synergistic effect between H_2O_2 and nitrite, leading to the generation of singlet oxygen and triggering of an autoamplificatory process, in which tumor cells, but not nonmalignant cells, were contributing to their own cell death through promotion of secondary singlet oxygen generation, catalase inactivation and reactivation of intercellular apoptosis-inducing ROS signaling. It presents experimentally derived data obtained with defined pure compounds that seem to explain a dominant path for the antitumor action of CAP and PAM. The significance of these data for induction of tumor cell apoptosis by application of CAP and PAM derived from a real plasma source has been meanwhile confirmed (Bauer et al., in preparation).

The data presented here do, however, not exclude the possible existence of other potential tumor cell death triggering compounds in PAM or CAP. Therefore we should not ignore potential roles of other ROS/RNS in this context.

Looking at PAM first, we realize that certain potential candidate compounds like singlet oxygen or ozone, derived from the gaseous phase of CAP, can be excluded as acting compounds in PAM, due to their extreme short life time which is based on their high reactivity. However, Kurake et al. (29) recognized, that nitrite and H_2O_2 applied in reconstitution experiments were excellently mimicking, and thus explaining the effect of PAM, but did not perfectly match in a quantitative way. Therefore, Kurake et al. (29) concluded that PAM should contain additional effectors to induce tumor cell death. These effectors acted obviously less dominant than the combination of nitrite and H_2O_2, and therefore have not yet been defined (29). Therefore, further investigation on additional candidate compounds in PAM are demanding. These studies should also include the search for reaction products between biomolecules and CAP-/PAM-derived ROS/RNS, which might have acquired signaling function after their reaction with ROS/RNS.

Looking at CAP, it is obvious that singlet oxygen derived from the gaseous phase of CAP should have the same triggering potential for tumor cell death as singlet oxygen generated from nitrite/H_2O_2 interaction. The limitation here, is the very high reactivity of singlet oxygen with biological material, which prevents singlet oxygen to reach its target cells when these are covered by medium *in vitro* or other tissue *in vivo*. An increase in singlet oxygen production by the plasma source, in combination with the experimental conditions (e. g. layer of medium or mode of application *in vivo*) might be useful for this issue. This consideration can be directly applied to the analysis of the potential effects of ozone and to the evaluation of conceivable synergistic effects established through the interaction between CAP-derived NO, peroxynitrite or NO_2 with singlet oxygen-mediated processes. In addition, it seems to be very promising to study in detail the effects of chlorine/chloride-related compounds in CAP, based on the connection between atomic oxygen and the formation and biological significance of hypochlorite/hypochlorous acid as shown by Bekeschus et al., [128], the multifaceted chemical biology of hypochlorite/hypochlorous acid and dichloride anion radicals as established by Wende et al. [129]. and the potential of hypochlorous acid to interfere with catalase activity, in addition to its potential to generate hydroxyl radicals through interaction with superoxide anions, as discussed by Krych-Madej and Gebicka [130].

These considerations show that despite defining one path for specific induction of tumor cell apoptosis by long-lived species in CAP and PAM, our study has not finalized the search for active ROS/RNS in CAP and PAM, but rather opened a new field of experimentation, with the goal to understand and optimize CAP and PAM applications in patients in the future.

Though the model of H_2O_2/nitrite-dependent selective apoptosis induction in tumor cells already contains an appreciable amount of complexity, it does by no means summarize the whole process of CAP and PAM-triggered antitumor effects *in vivo*. As CAP and PAM-mediated cell death has been characterized as immunogenic cell death [131–139] and as immunogenic cell death has been shown to act as a necessary

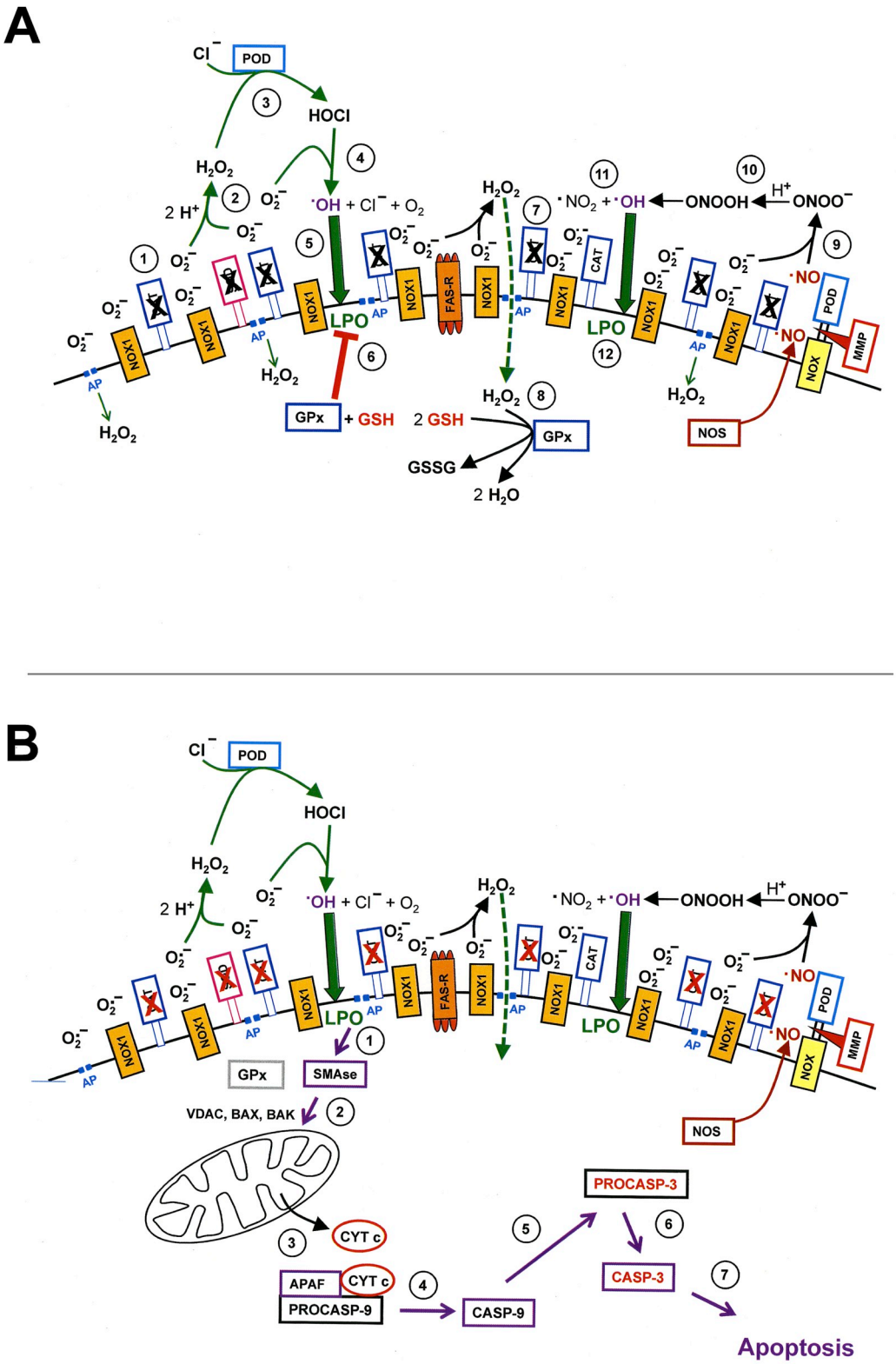

Fig. 20. Continuation of the processes described in Fig. 19

A. After sufficient inactivation of catalase molecules, the concentration of free H_2O_2 is sufficiently high to allow for HOCl formation through peroxidase (POD) (#1 - #3). HOCl then interacts with superoxide anions, resulting in the generation of hydroxl radicals (#4). This leads to lipid peroxidation (#5), which, at this points remains still without apoptosis-inducing effect due to the activity of glutathione peroxidase/GSH (#6). Due to extensive inactivation of catalase (#7), continuous intrusion of H_2O_2 causes further depletion of GSH (#7, #8). Under certain conditions, NO/peroxynitrite signaling will lead to lipid peroxidation (#9 - #12).

B. As soon as the intracellular GSH level is sufficiently depleted by constant influx of H_2O_2, lipid peroxidation has a chance to trigger the mitochondrial pathway of apoptosis (#1 - #7), as described in Bauer, 2017 a. Sphingomyelinase (SMAse), cytochrome C (Cyt c), APAF and procaspases/caspases-9 and -3 have essential functions during execution of apoptosis.

In vivo, the completion of these steps has a good chance to act as immunogenic cell death and to trigger a subsequent T cell-dependent antitumor mechanism.

final step in tumor therapy [140–145], cell death triggered by nitrite and H_2O_2 seems to first control a complex, selfsustaining and auto-amplificatory process selectively in tumor cells. This is then most likely triggering a likewise complex, intercalated and specific immunological process. The detailed knowledge on the initial process of selective cell death is hopefully instrumental to establish and to improve rational designs of tumor treatment by CAP and PAM in the future. In addition, these findings might hopefully open the path to rationally determine potential synergistic effects that should allow to increase the efficiency and selectivity of CAP and PAM treatment while lowering potential unwanted side effects.

This study defined a concentration range of H_2O_2 (approximately between 10 and 80 μM in which H_2O_2 caused selective apoptosis induction in tumor cells, provided it was applied together with nitrite, but not without nitrite. It was obvious that the clue for selective apoptosis induction in tumor cells, was to work with concentrations of H_2O_2 that are too low to induce apoptosis in nonmalignant cells, but high enough to establish the synergistic effect together with nitrite on tumor cells. This principle should be kept in mind for CAP and PAM applications that modulate the composition of CAP and PAM.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2019.101291.

References

[1] G. Bauer, Signal amplification by tumor cells: clue to the understanding of the antitumor effects of cold atmospheric plasma and plasma-activated medium, IEEE Trans. Radiat. Plasma Med. Sci. 2 (2018) 87–98.
[2] G. Bauer, Cold atmospheric plasma and plasma-activated medium: antitumor cell effects with inherent synergistic potential, Plasma Med. (2019), https://doi.org/10.1615/PlasmaMed.1019029462.
[3] D.B. Graves, The emerging role of reactive oxygen and nitrogen species in redox biology and some implications for plasma applications to medicine and biology, J. Phys. D Appl. Phys. 45 (2012) 263001, https://doi.org/10.1088/0022-3727/45/26/263001.
[4] D.B. Graves, Mechanisms of plasma medicine: coupling plasma physics, biochemistry, and biology, IEEE Trans. Radiat. Plasma Med. Sci. 1 (2017) 281–292.
[5] M. Keidar, R. Walk, A. Shashurin, P. Srinivasan, P. Sandler, A. Sandler, S. Dasgupta, R. Ravi, R. Guerrero-Preston, B. Trink, Cold plasma selectivity and the possibility of a paradigm shift in cancer therapy, Br. J. Canc. 105 (2011) 1295–1301.
[6] M. Keidar, A. Shashurin, O. Volotskova, M.A. Stepp, P. Srinivasan, A. Sandler, B. Trink, Cold atmospheric plasma in cancer therapy, Phys. Plasmas 20 (2013) 057101, , https://doi.org/10.1063/1.4801516.
[7] M. Keidar, Plasma for cancer treatment, Plasma Sources Sci. Technol. 24 (2015) 033001.
[8] M. Ishaq, M. Evans, K. Ostrikov, Effect of atmospheric gas plasmas on cancer cell signaling, Int. J. Cancer 134 (2013) 1517–1528, https://doi.org/10.1002/ijc.28323.
[9] N. Barekzi, M. Laroussi, Effects of low temperature plasmas on cancer cells, Plasma Process. Polym. 10 (2013) 1039–1050.
[10] M. Laroussi, From killing bacteria to destroying cancer cells: 20 years of plasma medicine, Plasma Process. Polym. 11 (2014) 1138–1141.
[11] M. Laroussi, Low-temperature plasma jet for biomedical applications: a review, IEEE Trans. Plasma Sci. 43 (2015) 703–712.
[12] J. Schlegel, J. Köritzer, V. Boxhammer, Plasma in cancer treatment, Clin. Plasma Med. 1 (2013) 2–7.
[13] H. Tanaka, M. Mizuno, K. Ishikawa, K. Takeda, K. Nakamura, F. Utsumi, H. Kajiyama, H. Kano, Y. Okazaki, S. Toyokuni, S. Maruyama, F. Kikkawa, M. Hori, Plasma medical Science for Cancer therapy: toward cancer therapy using nonthermal atmospheric pressure plasma, IEEE Trans. Plasma Sci. 42 (2013) 3760–3764, https://doi.org/10.1109/TPS.014.2353659.
[14] D.B. Graves, Reactive species from cold atmospheric plasma: implications for cancer therapy, Plasma Process. Polym. 11 (2014 a) 1120–1127.
[15] E.A. Ratovitski, X. Heng, D. Yan, J.H. Sherman, J. Canady, B. Trink, M. Keidar, Anti-Cancer Therapies of 21st century: novel approach to treat human cancers using cold atmospheric plasma, Plasma Process. Polym. 11 (2014) 1128–1137.
[16] P. Babington, K. Rajjoub, J. Canady, A. Siu, M. Keidar, J.H. Sherman, Use of cold atmospheric plasma in the treatment of cancer, Biointerphases 10 (2015) 029403.
[17] J. Gay-Mimbrera, M. Garcia MC, B.I. Tejera, A. Rodero-Serrano, A.V. Garcia-Nieto, J. Ruano, Clinical and biological principles of cold atmospheric plasma application in skin cancer, Adv. Ther. 33 (2016) 894–909.
[18] D.Y. Yan, J.H. Sherman, M. Keidar, Cold atmospheric plasma, a novel promising anti-cancer treatment modality, Oncotarget 8 (2017) 15977–15995.
[19] H.-R. Metelmann, D.S. Nedrelow, C. Seebauer, M. Schuster, T. von Woedtke, K.-D. Weltmann, S. Kindler, P. Metelmann PH, S.E. Finkelstein, D.D. Von Hoff, Head and neck cancer treatment and physical plasma, Clin. Plasma Med. 3 (2015) 17–23.
[20] H.-R. Metelmann, C. Seebauer, V. Miller, A. Fridman, G. Bauer, D.B. Graves, J.-M. Pouvesle, R. Rutkowski, M. Schuster, S. Bekeschus, K. Wende, K. Masur, S. Hasse, T. Gerling, M. Hori, H. Tanaka, E.H. Choi, K.-D. Weltmann, P.H. Metelmann, D.D. von Hoff, T. von Woedtke, Clinical experience with cold plasma in the treatment of locally advanced head and neck cancer, Clin. Plasma Med. 9 (2018) 6–13 http://doi.org/10.1016/j.cpme.2017.09.001.
[21] M. Schuster, C. Seebauer, R. Rutkowski, A. Hauschild, F. Podmelle, C. Metelmann, B. Metelmann, T. Von Woedtke, S. Hasse, K.-D. Weltmann, H.-R. Metelmann, Visible tumor surface response to physical plasma and apoptotic cell kill in head and neck cancer, Cranio Maxillo. Facial Surg. 44 (2016) 1445–1452.
[22] E.J. Szili, J.W. Bradley, R.D. Short, A "tissue model" to study the plasma delivery of reactive oxygen species, J. Phys. D-Appl. Phys. 47 (2014) 152002.
[23] E.J. Szili, J.S. Oh, S.H. Hong, A. Hatta, R.D. Short, Probing the transport of plasma-generated RONS in an agarose target as surrogate for real tissue: dependency on time, distance and material composition, J. Phys. D Appl. Phys. 48 (2015) 202001.
[24] E.J. Szili, S.-H. Hong, J.-S. Oh, N. Gaur, R.D. Short, Tracking the penetration of plasma reactive species in tissue models, Trends Biotechnol. 36 (2018 a) 594–602.
[25] E.J. Szili, J.S. Oh, H. Fukuhara, R. Bhatia, N. Gaur, C.K. Nguyen, S.H. Hong, S. Ito, K. Ogawa, C. Kawada, T. Shuin, M. Tsuda, M. Furikata, A. Kurabayashi, H. Furuta, M. Ito, K. Inoue, A. Hatta, R.D. Short, Modelling the helium plasma jet delivery of reactive species into a 3D cancer tumor, Plasma Sources Technol. 27 (2018) 014001.
[26] L. Nie, Y. Yang, J. Duan, F. Sun, X. Lu, G. He, Effect of tissue thickness and liquid composition on the penetration of long-lifetime reactive oxygen and nitrogen species (RONS) generated by plasma jet, J. Phys. D Appl. Phys. 51 (2018) 345204.
[27] A. Yan, N. Talbot, X. Nourmokammadi, J. Cheng, J. Canady, J.H. Sherman, M. Keidar, Principles of using cold atmospheric plasma stimulated media for cancer treatment, Sci. Rep. 5 (2015) 1833901–18339017.
[28] P.-M. Girard, A. Arbabian, M. Fleury, G. Bauville, PuechV, M. Dutreix, J.S. Sousa, Synergistic effect of H_2O_2 and NO_2^- in cell death induced by cold atmospheric He plasma, Sci. Rep. 6 (2016) 29098.
[29] N. Kurake, H. Tanaka, K. Ishikawa, T. Kondo, M. Sekine, K. Nakamura, H. Kajiyama, F. Kikkawa, M. Mizuno, M. Hori, Cell survival of glioblastoma grown in medium containing hydrogen peroxide and/or nitrite, or in plasma-activated medium, Arch. Biochem. Biophys. 605 (2016) 102–108.
[30] G. Uchida, A. Nakajima, K. Takenaka, T. Kawasaki, K. Koja, M. Shiratani, Y. Setsuhara, Effects of nonthermal plasma jet irradiation on the selective production of H_2O_2 and NO_2^- in liquid water, J. Appl. Phys. 120 (2016) 201102.
[31] H. Jablonowski, T. von Woedtke, Research on plasma medicine-relevant plasma-liquid interaction: what happened in the past five years? Clin. Plasma Med. 3 (2015) 42–52.
[32] F. Girard-Sahun, V. Badets, P. Lefrançois, N. Sojic, F. Clement, S. Arbault, Reactive Oxygen Species Generated by Cold Atmospheric Plasmas in Aqueous Solution: Successful Electrochemical Monitoring in Situ under a High Voltage System, (2019), https://doi.org/10.1021/acs.analchem.9b01912.
[33] P. Lukes, E. Dolezalova, I. Sisrova, M. Clupek, Aqueous-phase chemistry and bactericidal effects from an air discharge plasma in contact with water: evidence for the formation of peroxynitrite through a pseudo-second-order post-discharge reaction of H_2O_2 and HNO_2, Plasma Sources Sci. Technol. 23 (2014) 015019.
[34] H. Tanaka, K. Ishikawa, K. Nakamura, H. Kajiyama, H. Komo, T. Kikkawa, M. Hori, Plasma-activated medium selectively kills glioblastoma brain tumor cells by down-regulating a survival signaling molecule, AKT kinase, Plasma Med. 1 (2011) 265–277, https://doi.org/10.1615/PlasmaMed.2012006275.
[35] F. Utsumi, H. Kajiyama, K. Nakamura, H. Tanaka, M. Mizuno, K. Ishikawa, H. Kondo, H. Kano, M. Hori, F. Kikkawa, Effect of indirect nonequilibrium atmospheric pressure plasma on anti-proliferative activity against chronic chemoresistant ovarian cancer cells in vitro and in vivo, PLoS One 8 (2013), https://doi.org/10.1371/journal.pone.0081576 e8157601-e815760110, 2013.
[36] D. Yan, J.H. Sherman, X. Cheng, E. Ratovitski, J. Canady, M. Keidar, Controlling plasma stimulated media in cancer treatment application, Appl. Phys. Lett. 105 (2014) 22410101–22410104, https://doi.org/10.1063/1.4902875.
[37] T. Adachi, H. Tanaka, S. Nonomura, H. Hara, S.-I. Kondo, M. Hori, Plasma-activated medium induces A459 cell injury via a spiral apoptotic cascade involving the mitochondrial-nuclear network, Free Radic. Biol. Med. 79 (2015) 28–44.
[38] S. Mohades, M. Laroussi, J. Sears, N. Barekzi, H. Razavi, Evaluation of the effects of a plasma-activated medium on cancer cells, Phys. Plasmas 22 (2015) 122001.
[39] N. Kumar, J.H. Park, S.N. Jeon, B.S. Park, E.H. Chori, P. Attri, The action of microsecond -pulsed plasma-activated media on the inactivation of human lung cancer cells, J. Phys. D Appl. Phys. 49 (2016) 11540101–11540109, https://doi.org/10.1088/0022-3727/49/11/115401.

[40] D. Yan, N. Nourmohammadi, K. Bian, F. Murad, J.H. Sherman, M. Keidar, Stabilizing the cold plasma-stimulated medium by regulating medium's composition, Sci. Rep. 6 (2016) 2602, https://doi.org/10.1038/srep26016.

[41] D. Koensgen, I. Besic, D. Gumbel, A. Kaul, M. Weiss, K. Diesing, A. Kramer, S. Bekeschus, A. Mustea, M.B. Stope, Cold atmospheric plasma (CAP) and CAP-stimulated cell culture media suppress ovarian cancer cell growth – a putative treatment option in ovarian cancer therapy, Anticancer Res. 37 (2017) 6739–6744.

[42] S. Heinzelmann, G. Bauer, Multiple protective functions of catalase against intercellular apoptosis-inducing ROS signaling of human tumor cells, Biol. Chem. 391 (2010) 675–693.

[43] B. Böhm, S. Heinzelmann, M. Motz, G. Bauer, Extracellular localization of catalase is associated with the transformed state of malignant cells, Biol. Chem. 396 (2015) 1339–1356.

[44] G. Bauer, HOCl and the control of oncogenesis, J. Inorg. Biochem. 179 (2018 b) 10–23, https://doi.org/10.1016/j.jinorgbio.2017.11.005.

[45] G. Bauer, Nitric oxide contributes to selective apoptosis induction in malignant cells through multiple reaction steps, Crit. Rev. Oncog. 21 (2016) 365–398 2016.

[46] G. Bauer, Targeting the protective catalase of tumor cells with cold atmospheric plasma-treated medium (PAM), Anti Cancer Agents Med. Chem. 18 (2018 c) 784–804, https://doi.org/10.2174/1871520617666170801103708.

[47] G. Bauer, Autoamplificatory singlet oxygen generation sensitizes tumor cells for intercellular apoptosis-inducing signaling, Mech. Ageing Dev. 172 (2018) 59–77.

[48] G. Bauer, Tumor cell protective catalase as a novel target for rational therapeutic approaches based on specific intercellular ROS signaling, Anticancer Res. 32 (2012) 2599–2624.

[49] G. Bauer, Targeting extracellular ROS signaling of tumor cells, Anticancer Res. 34 (2014) 1467–1482.

[50] G. Bauer, Increasing the endogenous NO level causes catalase inactivation and reactivation of intercellular apoptosis signaling specifically in tumor cells, Redox Biol 6 (2015) 353–371.

[51] K. Irani, Y. Xia, J.L. Zweier, S.J. Sollott, C.J. Der, E.R. Fearon, M. Sundaresan, T. Finkel, P.J. Goldschmidt-Clermont, Mitogenic signalling by oxidants in Ras-transformed fibroblasts, Science 275 (1997) 1649–1652.

[52] K. Irani, P.J. Goldschmidt-Clermont, Ras, superoxide and signal transduction, Biochem. Pharmacol. 55 (1998) 1339–1346.

[53] M. Lopez-Lazaro, Excessive superoxide anion generation plays a key role in carcinogenesis, Int. J. Cancer 120 (2007) 1378–1380.

[54] M. Lopez-Lazaro, Dual role of hydrogen peroxide in cancer: possible relevance to cancer chemoprevention and therapy, Cancer Lett. 252 (2007) 1–8.

[55] W. Bechtel, G. Bauer, Catalase protects tumor cells against apoptosis induction by intercellular ROS signaling, Anticancer Res. 29 (2009) 4541–4557.

[56] W. Bechtel, G. Bauer, Modulation of intercellular ROS signaling of human tumor cells, Anticancer Res. 29 (2009) 4559–4570.

[57] M. Riethmüller, N. Burger, G. Bauer, Singlet oxygen treatment of tumor cells triggers extracellular singlet oxygen generation, catalase inactivation and reactivation of intercellular apoptosis-inducing signaling, Redox Biol 6 (2015) 157–168.

[58] G. Bauer, M. Motz, The antitumor effect of single-domain antibodies directed towards membrane-associated catalase and superoxide dismutase, Anticancer Res. 36 (2016) 5945–5956.

[59] G. Bauer, HOCl-dependent singlet oxygen and hydroxyl radical generation modulate and induce apoptosis of malignant cells, Anticancer Res. 33 (2013) 3589–3602.

[60] M. Herdener, S. Heigold, M. Saran, G. Bauer, Target cell-derived superoxide anions cause efficiency and selectivity of intercellular induction of apoptosis, Free Radic. Biol. Med. 29 (2000) 1260–1271.

[61] S. Heigold, C. Sers, W. Bechtel, B. Ivanovas, R. Schäfer, G. Bauer, Nitric oxide mediates apoptosis induction selectively in transformed fibroblasts compared to nontransformed fibroblasts, Carcinogenesis 23 (2002) 929–941.

[62] S. Pottgiesser, S. Heinzelmann, G. Bauer, Intercellular HOCl-mediated apoptosis induction in malignant cells: interplay between NOX1-dependent superoxide anion generation and DUOX-related HOCl-generating peroxidase activity, Anticancer Res. 35 (2015) 5927–5943.

[63] G. Bauer, Central signaling elements of intercellular reactive oxygen/nitrogen species-dependent induction of apoptosis in malignant cells, Anticancer Res. 37 (2017 a) 499–514.

[64] G. Bauer, SiRNA-based analysis of the abrogation of the protective function of membrane-associated catalase of tumor cells, Anticancer Res. 37 (2017) 567–582.

[65] L. Gebicka, J. Didik, Catalytic scavenging of peroxynitrite by catalase, Int. J. Inorg. Biochem. 103 (2009) 1375–1379.

[66] L. Brunelli, V. Yermilov, J.S. Beckman, Modulation of catalase peroxidatic and catalytic activity by nitric oxide, Free Rad. Biol. Med. 30 (2001) 709–714.

[67] D.A. Wink, J.B. Mitchell, Chemical biology of nitric oxide: insights into regulatory, cytotoxic, and cytoprotective mechanisms of nitric oxide, Free Radic. Biol. Med. 25 (1998) 434–456.

[68] A.S. Verkman, M. Hara-Chikuma, M.C. Papadopoulos, Aquaporins – new players in cancer biology, J. Mol. Med. 86 (2008) 523–529.

[69] G. Bauer, N. Zarkovic, Revealing mechanisms of selective, concentration-dependent potentials of 4-hydroxy-2-nonenal to induce apoptosis in cancer cells through inactivation of membrane-associated catalase, Free Radic. Biol. Med. 81 (2015) 128–144.

[70] J.F.R. Kerr, A.H. Wyllie, A.R. Currie, Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics, Br. J. Canc. 26 (1972) 239–257.

[71] S. Elmore, Apoptosis: a review of programmed cell death, Toxicol. Pathol. 35 (2007) 495–515.

[72] J. Jürgensmeier, C.P. Schmitt, E. Viesel, P. Höfler, G. Bauer, TGF-ß-treated normal fibroblasts eliminate transformed fibroblasts by induction of apoptosis, Cancer Res. 54 (1994) 393–398.

[73] E. Beck, R. Schäfer, G. Bauer, Sensitivity of transformed fibroblasts for intercellular induction of apoptosis is determined by their transformed phenotype, Exp. Cell Res. 234 (1997) 47–56.

[74] K. Scheit, G. Bauer, Direct and indirect inactivation of tumor cell protective catalase by salicylic acid and anthocyanidins reactivates intercellular ROS signaling and allows for synergistic effects, Carcinogenesis 36 (2015) 400–411.

[75] G. Bauer, S. Bereswill, P. Aichele, E. Glocker, Helicobacter pylori protects oncogenically transformed cells from reactive oxygen species-mediated intercellular induction of apoptosis, Carcinogenesis 35 (2014) 1582–1591.

[76] G. Bauer, D.B. Graves, Mechanisms of selective antitumor action of cold atmospheric plasma-derived reactive oxygen and nitrogen species, Plasma Process. Polym. 13 (2016) 1157–1178.

[77] H. Imai, Y. Nakagawa, Biological significance of phospholipid hydroperoxide glutathione peroxidase (PHGPx, GPx4) in mammalian cells, Free Radic. Biol. Med. 34 (2003) 145–169.

[78] B. Zucker, J. Hanusch, G. Bauer, Glutathione depletion in fibroblasts is the basis for induction of apoptosis by endogenous reactive oxygen species, Cell Death Differ. 4 (1997) 388–395.

[79] P.W. Gout, A.R. Buckley, C.R. Simms, N. Bruchovsky, Sulfasalazine, a potent suppressor of lymphoma growth by inhibition of the Xc^- cysteine transporter: a new action for an old drug, Leukemia 15 (2001) 1633–1640.

[80] J. Krych-Madej, L. Gebicka, Interactions of nitrite with catalase: enzyme activity and reaction kinetic studies, J. Inorg. Biochem. 17 (2017) 10–17.

[81] D.Y. Yan, A. Talbot, N. Nourmohammadi, J.H. Sherman, X.Q. Cheng, M. Keidar, Toward understanding the selective anticancer capacity of cold atmospheric plasma- A model based on aquaporins, Biointerphases 10 (2015) 040801.

[82] D. Yan, H. Xiao, W. Zhu, N. Nourmohammadi, L.G. Zhang, K. Bian, M. Keidar, The role of aquaporins in the anti-glioblastoma capacity of the cold plasma-stimulated medium, J. Phys. D Appl. Phys. 50 (2017) 055401.

[83] J. Van der Paal, C. Verheyen, E.C. Neyts, A. Bogaerts, Hampering effect of cholesterol on the permeation of reactive oxygen species through phospholipid bilayer: possible explanation for plasma cancer selectivity, Sci. Rep. 7 (2017) 39526.

[84] W. Van Boxem, J. Van der Paal, Y. Gorbanev, S. Vanuytsel, E. Smits, S. Dewilde, A. Bogaerts, Anti-cancer capacity of plasma-treated PBS: effect of chemical composition on cancer cell cytotoxicity, Sci. Rep. 7 (2017) 16478, https://doi.org/10.1038/s41598-017-16758-8.

[85] P. Di Mascio, E.J.H. Bechara, M.H.G. Medeiros, K. Briviba, H. Sies, Singlet molecular oxygen production in the reaction of peroxynitrite with hydrogen peroxide, FEBS Lett. 355 (1994) 287–289.

[86] J.A. Escobar, A. Rubio, E.A. Lissi, SOD and catalase inactivation by singlet oxygen and peroxyl radicals, Free Radic. Biol. Med. 20 (1996) 285–290.

[87] Y.K. Kim, O.J. Kwon, J.-W. Park, Inactivation of catalase and superoxide dismutase by singlet oxygen derived from photoactivated dye, Biochimie 83 (2001) 437–444.

[88] F. Alvarez, A. Denicola, R. Radi, Reaction between peroxynitrite and hydrogen peroxide: formation of oxygen and slowing of peroxynitrite decomposition, Chem. Res. Toxicol. 8 (1995) 859–864.

[89] S. Goldstein, J. Lind, G. Merenyi, Chemistry of peroxynitrites as compared to peroxynitrates, Chem. Rev. 105 (2005) 2457–2470.

[90] S. Miyamoto, G.E. Ronsein, T.C. Correa, G.R. Martinez, M.H.G. Medeiros, P. Di Mascio, Direct evidence of singlet molecular oxygen generation form peroxynitrate, a decomposition product of peroxynitrite-, Dalton Trans. 29 (2009) 5720–5729, https://doi.org/10.1039/b905560f 2009.

[91] J.S. Beckman, T.W. Beckman, J. Chen, P.A. Marshall, B.A. Freeman, Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury form nitric oxide and superoxide, Proc. Natl. Acad. Sci. U.S.A. 87 (1990) 1620–1624.

[92] G. Merenyi, J. Lind, S. Goldstein, G. Czapski, Peroxynitrite homolyzes into $^{\cdot}OH$ and $^{\cdot}NO_2$ radicals, Chem. Res. Toxicol. 11 (1998) 712–713.

[93] H. Christensen, K. Sehested, H. Corfitzen, Reactions of hydroxyl radicals with hydrogen peroxide at ambient and elevated temperature, J. Phys. Chem. 86 (1982) 1588–1590.

[94] F. Girard, M. Peret, N. Dumont, V. Badets, S. Blanc, K. Gazelik, C. Noel, T. Belmonte, L. Martin, J.-P. Cambus, G. Simon, N. Sojic, B. Held, S. Arbault, F. Clémon, Correlations between gaseous and liquid phase chemistries induced by cold atmospheric plasmas in physiological buffer, Phys. Chem. Chem. Phys. 20 (2018) 9198–9210.

[95] K. Kitano, S. Ikawa, Y. Nakashima, N. Kawashita, A. Tani, Peroxynitric acid (HOONO2) is the active component in cryo-preserved plasma-treated water with reduced-pH method for effective and safety disinfection, ISPC Conference, Pittsburgh, Pennsylvania, 2017.

[96] S. Ikawa, A. Tani, Y. Nakashima, K. Kitano, Physico-chemical properties of bactericidal plasma-treated water, J. Phys. D Appl. Phys. 49 (2016) 425401.

[97] Z.C. Liu, D.X. Liu, c Chen, Z.J. Liu, A.J. Yang, M.Z. Rong, H.L. Chen, M.G. Kong, Post-discharge evolution of reactive species in the water activated by a surface air plasma: a modeling study, J. Phys. D Appl. Phys. 51 (2018) 175202.

[98] A.B.A. Denicola, B.A. Freeman, M. Trujillo, R. Radi, Peroxynitrite reaction with carbon dioxide/bicarbonate: kinetics and influence on peroxynitrite-mediated reactions, Arch. Biochem. Biophys. 333 (1996) 49–58.

[99] S. Goldstein, G. Czapski, Formation of peroxynitrate from the reaction of peroxynitrite with CO_2: evidence for carbonate radical production, J. Am. Chem. Soc. 120 (1998) 3458–3463.

[100] G.L. Squadrito, W.A. Pryor, Oxidative chemistry of nitric oxide: the roles of superoxide, peroxynitrite, and carbon dioxide, Free Radic. Biol. Med. 25 (1998)

392–403.
[101] I. Fridovich, Superoxide dismutases, Annu. Rev. Biochem. 44 (1975) 147–159.
[102] M. Saran, C. Michel, W. Bors, Reaction of NO with O_2^-. Implication for the action of endothelium-derived relaxing factor (EDRF), Free Rad. Res Commun. 10 (1990) 221–226.
[103] R.E. Huie, S. Padmaja, The reaction of NO with superoxide, Free Radic. Res. Commun. 18 (1993) 195–199.
[104] S. Goldstein, G. Czapski, The reaction of $^{\cdot}NO$ with $O_2^{\cdot-}$ and $HO_2^{\cdot}$: a pulse radiolysis study, Free Radic. Biol. Med. 19 (1995) 505–510.
[105] S. Zhuang, J.T. Demir, I.E. Kochevar, Protein kinase C inhibits singlet oxygen-induced apoptosis by decreasing caspase-8 activation, Oncogene 20 (2001) 6764–6776.
[106] Y. Suzuki, Y. Ono, Y. Hirabayashi, Rapid and specific reactive oxygen species generation via NADPH oxidase activation during FAS-mediated apoptosis, FEBS Lett. 425 (1998) 209–212.
[107] R. Reinehr, S. Becker, A. Eberle, S. Grether-Beck, D. Häussinger, Involvement of NADPH oxidase isoforms and src family kinases in CD95-dependent hepatocyte apoptosis, J. Biol. Chem. 280 (2005) 27179–27194.
[108] C. Selleri, T. Sato, A.M. Raiola, B. Rotoli, N.S. Young, J.P. Maciejewski, Induction of nitric oxide synthase is involved in the mechanism of FAS-mediated apoptosis in hematopoietic cells, Br. J. Hematol. 99 (1997) 481–489.
[109] L.W. Aurand, N.N. Boone, G.G. Giddings, Superoxide and singlet oxygen in milk lipid peroxidation, J. Dairy Sci. 60 (1977) 363–369.
[110] W.H. Koppenol, J. Butler, Mechanism of reactions involving singlet oxygen and the superoxide anion, FEBS (Fed. Eur. Biochem. Soc.) Lett. 83 (1977) 1–6.
[111] N.I. Krinsky, Singlet oxygen in biological systems, TIBS (Trends Biochem. Sci.) 2 (1977) 35–38.
[112] E.A. Mayeda, A.J. Bard, Singlet oxygen. The suppression of its production in dismutation of superoxide ion by superoxide dismutase, J. Am. Chem. Soc. 96 (1974) 4023–4024.
[113] E.J. Corey, M.M. Mehrotra, A.U. Khan, Water induced dismutation of superoxide anion generates singlet molecular oxygen, Biochem. Biophys. Res. Commun. 145 (1987) 842–846.
[114] A.U. Khan, M. Kasha, Singlet molecular oxygen in the Haber-Weiss reaction, Proc. Natl. Acad. Sci. U.S.A. 91 (1994) 12365–12367.
[115] E.D. Kerver, I.M.C. Vogels, K.S. Bosch, H. Vreeling-Sindelarova, R.J.M. van den Munckhof, W.M. Frederiks, In situ detection of spontaneous superoxide anion and singlet oxygen production by mitochondria in rat liver and small intestine, Histochem. J. 29 (1997) 229–237.
[116] M.J. Steinbeck, A.U. Khan, M.J. Karnovsky, Extracellular production of singlet oxygen by stimulated macrophages quantified using 9,10-diphenylanthracene and perylene in a polystyrene film, J. Biol. Chem. 268 (1993) 15649–15654.
[117] M. Tarr, D.P. Valenzeno, Singlet oxygen: the relevance of extracellular production mechanisms to oxidative stress in vivo, Photochem. Photobiol. Sci. 2 (2003) 355–361.
[118] T.P.A. Devasagayam, J.P. Kamat, Biological significance of singlet oxygen, Indian J. Exp. Biol. 40 (2002) 680–692.
[119] L.-O. Klotz, K.-D. Kröncke, H. Sies, Singlet oxygen-induced signaling effects in mammalian cells, Photochem. Photobiol. Sci. 2 (2003) 88–94.
[120] J.A. Badway, M.L. Kanovsky, Active oxygen species and the functions of phagocytic leukocytes, Annu. Rev. Biochem. 49 (1980) 695–726.
[121] J.R. Kanofsky, Singlet oxygen production by biological systems, Chem. Biol. Interactions 70 (1989) 1–28.
[122] D.F. Evans, M.W. Upton, Studies on singlet oxygen generation in aqueous solution. Part 4. The spontaneous and catalysed decomposition of hydrogen peroxide, J. Chem. Soc. Dalton Trans. 12 (1985) 2525–2529.
[123] H. Wu, P. Sun, H. Feng, H. Zhou, R. Wang, Y. Liang, J. Lu, W. Zhu, J. Zhang, J. Fang, Reactive oxygen species in a nonthermal plasma microjet and water system: generation, conversion and contributions to bacteria inactivation-an analysis by electron spin resonance spectroscopy, Plasma Process. Polym. 9 (2012) 417–424.
[124] L.P. Candeias, K.B. Patel, M.R.L. Stratford, P. Wardmann, Free hydroxyl radicals are formed on reaction between the neutrophil-derived species superoxide anion and hypochlorous acid, FEBS 333 (1993) 151–153.
[125] L.K. Folkes, L.P. Candeias, P. Wardman, Kinetics and mechanisms of hypochlorous acid reactions, Arch. Biochem. Biophys. 323 (1995) 120–126.
[126] S. Bekeschus, A. Mueller, V. Miller, U. Gaipl, K.-D. Weltmann, Physical plasma elicits immunogenic cancer cell death and mitochondrial singlet oxygen, IEE Trans. Radiat. Plasma Med. Sci. 2 (2018) 138–147, https://doi.org/10.1109/TRPMS.2017.2766027.
[127] A.M. Held, D.J. Halko, J.K. Hurst, Mechanisms of chlorine oxidation of hydrogen peroxide, J. Am. Chem. Soc. 100 (1978) 5732–5740.
[128] S. Bekeschus, K. Wende, M.M. Hefny, K. Rödder, H. Jablonowski, A. Schmidt, T. von Woedtke, K.-D. Weltmann, J. Benedikt, Oxygen atoms are critical in rendering THP-1 leukaemia cells susceptible to cold atmospheric plasma-induced apoptosis, Sci. Rep. 7 (2017) 2791.
[129] K. Wende, P. Williams, J. Dalluge, W. Van Gaens, H. Akoubakr, J. Bischof, T. von Woedtke, S.M. Goyal, K.-D. Weltmann, A. Bogaerts, K. Masur, P.J. Bruggeman, Identification of biologically active liquid chemistry induced by nonthermal atmospheric pressure plasma jet, Biointerphases 10 (2015) 029518, , https://doi.org/10.1116/1.4919710.
[130] J. Krych-Madej, L. Gebicka, Do pH and flavonoids influence hypochlorous acid-induced catalase inhibition and heme modification? Int. J. Biol. Macromol. 80 (2015) 162–169.
[131] A. Lin, B. Truong, A. Pappas, L. Kirifides, A. Oubarri, S. Chen, S. Lin, D. Dobrynin, G. Fridman, A. Fridman, N. Sang, V. Miller, Uniform nanosecond pulsed dielectric barrier discharge plasma enhances anti-tumor effects by induction of immunogenic cell death in tumors and stimulation of macrophages, Plasma Process. Polym. 12 (2015) 1392–1399.
[132] A. Lin, B. Truong, S. Patel, N. Kaushik, E.H. Choi, G. Fridman, A. Fridman, V. Miller, Nanosecond-pulsed DBD plasma-generated reactive oxygen species trigger immunogenic cell death in A549 lung carcinoma cells through intracellular oxidative stress, Int. J. Mol. Sci. 18 (2017) 966.
[133] A. Lin, B. Truong, G. Fridman, A. Fridman, V. Miller, Immune cells enhance selectivity of nanosecond-pulsed DBD plasma against tumor cells, Plasma Med. 7 (2017) 85–96.
[134] A.G. Lin, B. Xiang, D.J. Merlino, T.R. Baybutt, J. Sahu, A. Fridman, A.E. Snook, V. Miller, Non-thermal plasma induces immunogenic cell death in vivo in murine CT26 colorectal tumors, OncoImmunology 7 (2018) e148978.
[135] V. Miller, A. Lin, A. Fridman, Why target immune cells for plasma treatment of cancer, Plasma Chem. Plasma Process. 36 (2016) 259–268.
[136] K. Mizuno, Y. Yonetamari, Y. Shirakawa, T. Akiyama, R. Ono, Anti-tumor immune response induced by nanosecond pulsed streamer discharge in mice, J. Phys. D Appl. Phys. 50 (2017) 12LT01.
[137] S. Bekeschus, A. Mueller, V. Miller, U. Gaipl, K.-D. Weltmann, Physical plasma elicits immunogenic cancer cell death and mitochondrial singlet oxygen, IEE Trans. Radiat. Plasma Med. Sci. 2 (2018) 138–147.
[138] S. Bekeschus, R. Clemen, H.-R. Metelmann, Potentiating anti-tumor immunity with physical plasma, Clin. Plasma Med. 12 (2018) 17–22.
[139] N.K. Kaushik, N. Kaushik, B. Min, K. Choi KH, Y.J. Hong, V. Miller, A. Fridman, E.H. Choi, Cytotoxic macrophage-released tumour necrosis factor-alpha (TNF-alpha) as a killing mechanism for cancer cell death after cold plasma activation, J. Phys. D Appl. Phys. 49 (2018) 084001.
[140] L. Apetoh, A. Tesnier, F. Ghiringhelli, G. Kroemer, L. Zitvogel, Interactions between dying tumor cells and the innate immune system determine the efficiency of conventional antitumor therapy, Cancer Res. 68 (2008) 4026–4030.
[141] D.R. Green, T. Ferguson, L. Zitvogel, G. Kroemer, Immunogenic and tolerogenic cell death, Nat. Rev. Immunol. 9 (2009) 353–363.
[142] D.V. Krysko, A.D. Garg, A. Kaczmarek, O. Krysko, P. Agostinis, P. Vandenabeele, Immunogenic cell death and DAMPs in cancer therapy, Nat. Rev. Cancer 12 (2012) 860–875.
[143] G. Kroemer, L. Galluzzi, O. Kepp, L. Zitvogel, Immunogenic cell death in Cancer therapy, Annu. Rev. Immunol. 31 (2013) 51–72.
[144] A.D. Garg, P. Agostinos, ER stress, autophagy and immunogenic cell death in photodynamic therapy-induced anti-cancer immune responses, Photochem. Photobiol. Sci. 13 (2014) 474–487.
[145] S.M. Candeias, U.S. Gaipl, The immune system in cancer prevention, development and therapy, Anti Cancer Agents Med. Chem. 16 (2016) 101–107.